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Functional analysis and overexpression of a Glycogen Synthase Kinase-3β gene in *Lotus japonicus* during plant interaction with rhizobia

Λειτουργική ανάλυση και υπερέκφραση του γονιδίου GSK3β του *Lotus japonicus* κατά την αλληλεπίδραση του φυτού με ριζόβια

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Περιεχόμενα

Ευχαριστίες…

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Abstract

In mammals there are proteins known to get involved in many developmental and physiological procedures and are named Glycogen Synthase Kinases 3β (GSK3β). The same seems to apply in plants even though there are not as many information on that field. Through the present thesis two *Lotus japonicus* GSK3β-like kinases were identified and one of them was subjected to further experiments. Both the kinases isolated were full length on the databases used. One of them, (*LjGSK3β*), codes for a homologue of ASKθ in *Arabidopsis thaliana* providing this way an early confirmation for its characterization as GSK kinase. In *A. thaliana* ASKθ functions as a negative regulator of the Brassinosteroid signaling pathway (Rozhon *et al.,* 2010). The second full length sequence (*LjT36I04*) presents a high percentage of homology to the characterized ASKα of *A. thaliana* which is implicated in redox stress responses (Dal Santo *et al.,* 2012). Analysis using quantitative real time PCR showed that *LjGSK3β* is induced in the roots one hour post inoculation with *M.loti* and downregulated after 48 hours, compared to uninfected roots, thus suggesting a role of the kinase in the establishment of symbiotic relationship between legume and rhizobium. Furthermore, in an attempt to characterize the structural properties and also the functional activity of *Lj*GSK3β, a series of experiments were carried in presence of lupeol. Lupeol was shown to bind directly to mammalian GSK3β and regulate its kinase activity in pathways involved in cell proliferation (Harish *et al.,* 2008; Saleem *et al.,* 2009). In order to investigate the suggested interaction between lupeol and *Lj*GSK3β, the protein of *LjGSK3β* was overexpressed, isolated and purified and lupeol along with other terpenes were applied directly on the roots of growing plants both in presence and absence of *M.loti*. Using the purified protein, a photometric autophosphorylation assay was held in presence of lupeol resulting in decreased autphosphorylating activity on behalf of *Lj*GSK3β. As for the experiment with terpenes quantitative real time PCR on the treated roots followed, revealing fluctuations on the expression levels not only of *LjGSK3β* but also of other genes (*LjPUB13* and *LjSERK3*) thought to be involved in the symbiotic pathway of

L.japonicus with rhizobia. These results could suggest an implication of *LjGSK3β* as a regulatory molecule between pathways involved in symbiotic relationship establishment and hormone signaling both of which bear similarities to those of *A.thaliana*.

Περίληψη

Στα θηλαστικά είναι γνωστή η ύπαρξη πρωτεϊνών οι οποίες εμπλέκονται σε αρκετές αναπτυξιακές και λειτουργικές διαδικασίες και ονομάζονται Κινάσες της Συνθάσης του Γλυκογόνου 3β (GSK3β). Το αυτό φαίνεται να ισχύει και στα φυτά αν και οι πληροφορίες πάνω σε αυτόν τον τομέα είναι λίγες. Μέσα από την παρούσα πτυχιακή απομονώθηκαν δύο GSK3β κινάσες του φυτού *Lotus japonicus* εκ των οποίων η μία υποβλήθηκε σε περαιτέρω πειράματα. Στις ηλεκτρονικές βάσεις δεδομένων που χρησιμοποιήθηκαν, βρέθηκε και για τις δύο κινάσες οι πλήρους μήκους αλληλουχίες τους. Η μία κινάση (που θα ονομάζεται για λόγους ευχρηστίας *LjGSK3β,* κωδικοποιεί για μία ομόλογη πρωτεΐνη της κινάσης ASKθ του φυτού *Arabidopsis thaliana*, παρέχοντάς μας μία επιβεβαίωση για το σωστό χαρακτηρισμό της ως GSK3β κινάση. Στο *A. thaliana* η ASKθ λειτουργεί ως αρνητικός ρυθμιστής του σηματοδοτικού μονοπατιού των βρασσινοστεροειδών (Rozhon *et al.,* 2010). Η δεύτερη πλήρους μήκους αλληλουχία (*LjT36I04*) παρουσιάζει υψηλά ποσοστά ομολογίας με την χαρακτηρισμένη πρωτεΐνη ASKα του *Arabidopsis thaliana* η οποία εμπλέκεται στην απόκριση σε οξειδωτικό στρες (Dal Santo *et al.,* 2012). Ανάλυση με ποσοτική PCR πραγματικού χρόνου έδειξε ότι η LjGSK3β επάγεται στις ρίζες μία ώρα μετά τη μόλυνση με *M.loti* και μειώνεται μετά από 48 ώρες σε σχέση με τα επίπεδα έκφρασης σε μη μολυσμένα φυτά. Το γεγονός αυτό υποδηλώνει ένα πιθανό ρόλο της κινάσης στην εγκαθίδρυση της συμβιωτικής σχέσης μεταξύ ψυχανθών-ριζοβίων. Επιπρόσθετα, σε μια προσπάθεια να διασαφηνιστούν οι δομικές ιδιότητες αλλά και η λειτουργική ικανότητα της *Lj*GSK3β, πραγματοποιήθηκε μία σειρά πειραμάτων παρουσία λουπεόλης. Η λουπεόλη έχει αποδειχθεί ότι προσδένεται απευθείας στην GSK3β των θηλαστικών και ρυθμίζει την δραστηριότητα της κινάσης πάνω σε μονοπάτια που εμπλέκονται στον κυτταρικό πολλαπλασιασμό (Harish *et al.,* 2008; Saleem *et al.,* 2009). Για να μπορέσει να διερευνηθεί η προτεινόμενη αυτή αλληλεπίδραση μεταξύ της λουπεόλης και της GSK3β του *Lotus japonicus*, έγινε υπερέκφραση της πρωτεΐνης *Lj*GSK3β καθώς και απομόνωσή της σε καθαρή μορφή. Ακολούθησε επίσης πείραμα προσθήκης λουπεόλης και άλλων τερπενίων

απευθείας επάνω στη ρίζα φυτών, παρουσία και απουσία μόλυνσης από το *M.loti* ώστε να μελετηθεί σε μεταφραφικό επίπεδο τυχόν αλληλεπίδραση. Με βάση την καθαρή πρωτεΐνη, πραγματοποιήθηκε μία φωτομετρική δοκιμασία αυτοφωσφορυλίωσής της παρουσία λουπεόλης, γεγονός που οδήγησε σε μειωμένη ικανότητα αυτοφωσφορυλίωσης της *Lj*GSK3β. Όσο για το πείραμα με την προσθήκη τερπενίων, η ποσοτική PCR πραγματικού χρόνου πάνω σε δείγματα ριζών αποκάλυψε διακυμάνσεις τόσο στα επίπεδα έκφρασης της *LjGSK3β* όσο και στα επίπεδα γονιδίων όπως είναι τα *LjPUB13* and *LjSERK3* τα οποία φαίνεται να λαμβανάνουν μέρος στο σηματοδοτικό μονοπάτι υπεύθυνο για τη συμβίωση μεταξύ *L.japonicus* και ριζοβίων. Τα αποτελέσματα αυτά υποδηλώνουν μία πιθανή εμπλοκή της *Lj*GSK3β, σε ρόλο μοριακού ρυθμιστή μονοπατιών του *L.japonicus* υπεύθυνων τόσο για την εγκαθίδρυση της συμβιωτικής σχέσης όσο και για την απόκριση του φυτού σε ορμόνες.

1.Introduction

1.1 Function and Structure of mammalian GSK

GSK3 protein was characterised as a serine/threonine kinase responsible for the phosphorylation of glycogen synthase as a part of the animal insulin signaling pathway (Cohen *et al., 1982).* Since then, GSK3 has been one of the most studied molecules. There are two GSK3 genes, GSK3a and GSK3β. GSK3β can produce two different proteins GSK3β1 and GSK3β2 through alternative splicing. The catalytic domain is highly conserved between all GSK3 isoforms, although GSK3β2 has a 13 amino acid insertion in this domain. GSK3α has an N-terminal glycine rich extension that results in a larger relative molecular weight (51 kDa for GSK3α, and 47 kDa for GSK3β1, GSK3β2 has a molecular weight of around 42kDa). GSK3β isoform is expressed in every tissue although the expression pattern differs in each tissue whereas GSK3α is expressed mainly in neurons.

The structure of GSK3β consists of an amino-terminal β-sheet domain, coupled to a carboxy-terminal α -helical domain. The protein contains an N-terminal domain, a kinase domain and a C-terminal domain. Phosphorylation of Tyr216 located in the T-loop (activation site) facilitates substrate phosphorylation by GSK3 but is not strictly required for its kinase activity. Phosphorylation of GSK3B at Ser9 in N-terminal region leads to inhibition of its kinase activity (Figure 1). Binding domain (BD) includes GSK3 specific binding sites for substrates and protein complexes. Unlike many protein kinases involved in signal transduction, GSK3 is active in absence of signal and is inhibited in response to signals from the membrane-bound receptor Wnt/Fz (Dajani *et al.,* 2001).

Phosphorylation by GSK3 usually occurs after the substrate has already been phopshorylated in specific sites (primed phosphorylation) providing with the appropriate binding structure for further GSK3 phosphorylation (Doble and Woodgett *et al.,* 2003). The general GSK3 substrate consensus sequence is Ser/ThrXXX (PhosphoSer/Thr), where X is any residue. However, proposed substrates

of GSK3 exist that do not conform to this sequence, having either a priming site much further from the target site, or no apparent requirement for priming at all.

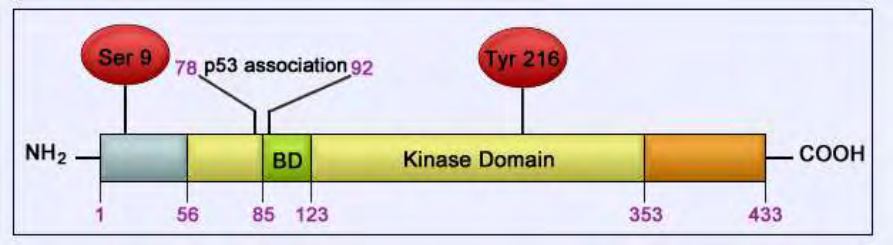


Figure 1: *GSK3β structure containing as a substrate example p53 protein binding site*

1.2 Roles of GSK3

1.2.1 Insulin pathway

As mentioned, GSK was first characterized as a part of insulin pathway. Indeed phosphorylation of glycogen synthase by GSK3 inactivates the synthase. Initial priming of glycogen synthase is undertaken by CKII at Ser656 enabling the phopshorylation by GSK3 at a series of site. Each additional phopshorylation has an additive effect on the inhibition.

1.2.2 Wnt/β-catenin pathway

After its implication within the insulin pathway, GSK was found to be among the main molecules participating in Wnt signaling pathway (Figure 2). In particular, when extracellular Wnt proteins bind to cell surface receptors such as FZD and LRP, GSK3 is phosphorylated leading to subsequent inhibition of β-catenin phosphorylation and thus resulting to its accumulation. Accumulated β-catenin is translocated in order to further interact with transcription factors and regulate gene expression (Hwang *et al.,* 2009).

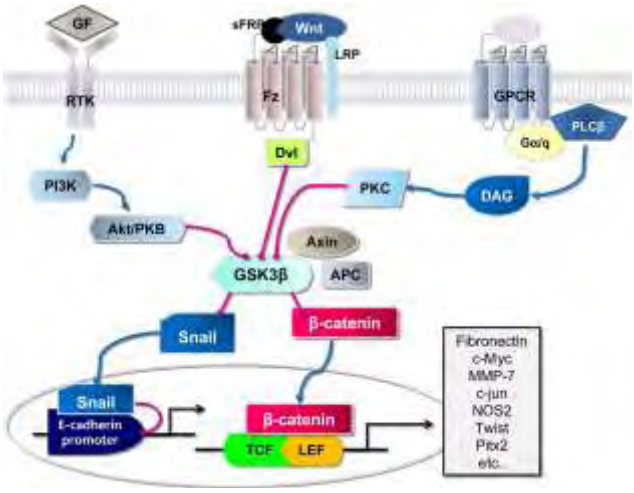


Figure 2: *A review of Wnt/β-catenin pathway*

1.2.3 Hedgehog pathway

Hedgehog (Hh) pathway controls both the development of *Droshophila* as well as limp development in vertebrates. A molecule known as Ci (Cubitus interruptus) is responsible for preventing inappropriate Hh pathway activation. Ci is downregulated by phosphorylation from three kinases, PKA, GSK3 and CK1. Also an F-box protein, β-TRCP that functions as a substrate recognition component of the SCF family of E3 ubiquitin ligases is part of the complex that regulates Ci (Chen and Jiang *et al.,* 2013)

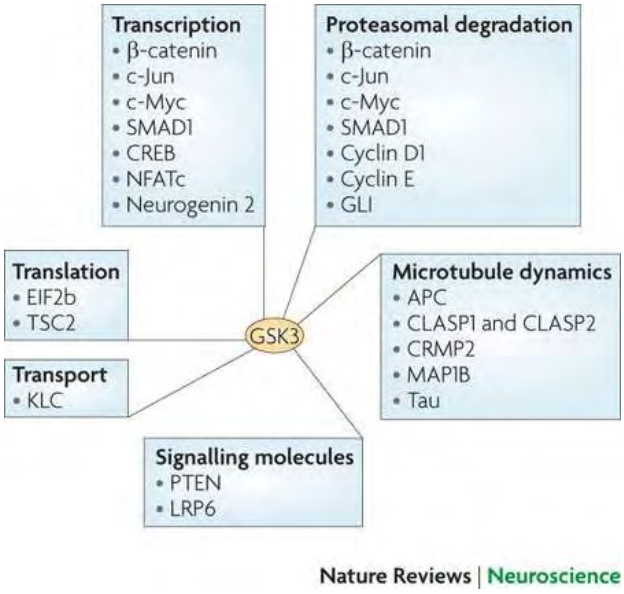


Figure 3: *GSK3 putative substrates.*

1.3 Pathological effects of abnormal GSK3 activity

The signaling pathways that GSK3 is involved regulate cell fate and morphology making GSK3 an important molecule in human diseases such as cancer, non-insulin-dependent diabetes mellitus and neurological pathologies as Alzheimer's and bipolar disorder. Given the association of abnormal GSK3 activity and its relevance to human pathophysiology (Figure 4), GSK3 has emerged as a potential therapeutic target. Several new small molecules that act as GSK-inhibitors and are ATP competitive have been recently developed (Martinez *et al.,* 2002b) (Coghlan *et al.,* 2000). Also non-ATP competitive molecules are starting to occur with the first of them being small heterocyclic thiadiazolidinones (TDZD) (Martinez *et al.,* 2002a) and later on halomethylketone (HMK) derivatives (Conde *et al.,* 2003).

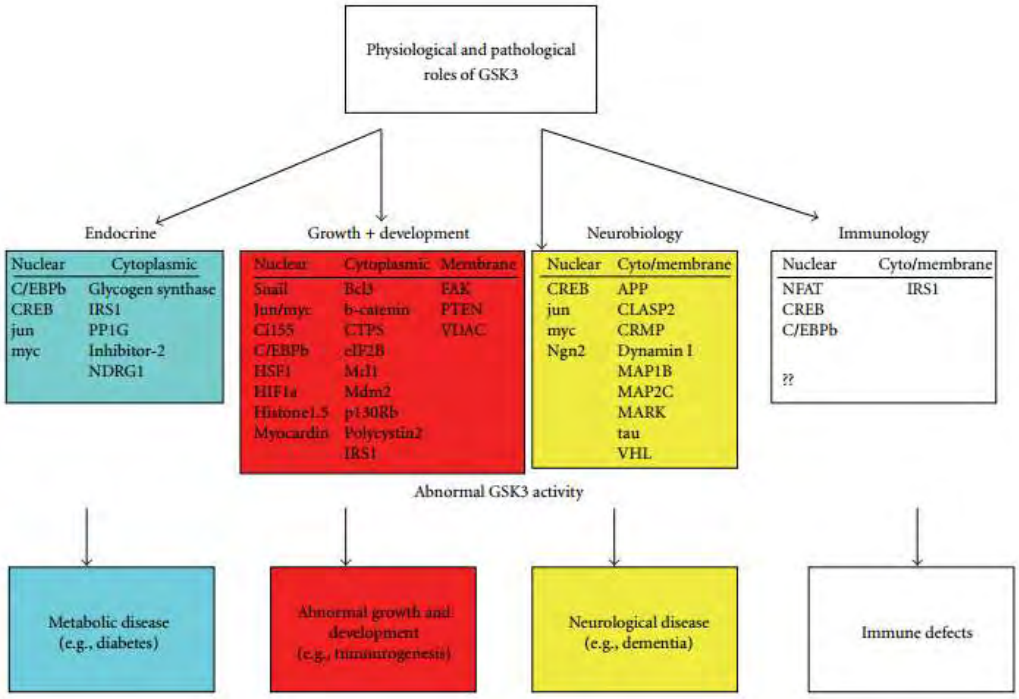


Figure 4: *Potential physiological and pathological effects of GSK3 activity*

1.4 Terpenes as natural inhibitors of GSK3

Besides synthetic inhibitors of GSK3, research has been focused on a natural category of molecules known as triterpenes. Triterpenoids are synthesized by the cyclization of squalene and are a group of natural compounds that serve as structural components of plant membranes stabilizing the phospholipid layers (Liby *et al.,* 2007). They are natural elements of everyday dietary and can be found in fruits, vegetables, cereals and vegetable oils. During the last years the interest for isolating terpenes and transforming them into active therapeutic agents has been increased since many of them have exhibited profound pharmacological responses against human diseases. One such triterpene that is known to implicate in GSK3 function is lupeol (Harish et al, 2008).

1.5 Lupeol and its effect on human diseases

The cyclization of 2,3-oxidosqualene by oxidosqualene cyclases (OSCs) leads, through the activity of lupeol synthase to the pentacyclic triterpene lupeol (Abe, Rohmer, Prestwich *et al.,* 1993). Lupeol possesses strong antioxidant, anti-inflammatory, antiarthritic, antimutagenic, and antimalarial activities in both *in vitro* and *in vivo* systems. Acts as a potent inhibitor of protein kinases and serine proteases and inhibits the activity of DNA topoisomerase II, a known target for anticancer chemotherapy (Saleem *et al.,* 2001, Hasmeda *et al.,* 2003, Rajic *et al.,* 2000).

There is a wide research field focused on lupeol and its role on human diseases. Lately lupeol was reported to promote wound healing in rat models by decreasing the level of monocytes and inhibiting GSK3β (Harish *et al.,* 2008). Another important characteristic of lupeol is its antitumor effect. In different types of cancer lupeol seems to have an effect upon a variety of signaling pathways. Among the pathways affected the main are nuclear factor kappa B (NFkB) and phosphatidylinositol 3-kinase PI3K/Akt (protein kinase B pathway) which are actively involved in tumorigenesis. In human melanoma, lupeol has been shown to inhibit growth of highly metastatic tumors by modulating ratio of Bcl-2 and Bax protein levels (Saleem *et al.,* 2008). Finally, in prostate cancer lupeol was observed to target directly or indirectly axin, GSK3β, MMP-2, ERBB-2 and c-myc. (Saleem *et al.,* 2009). Moreover in prostate cancer, where the complex GSK3β and axin is defective, lupeol increases

phosphorylation of β-catenin leading to its proteasomal degradation. Cells treated with lupeol demonstrated increased GSK3β protein levels and increased protein levels of axin and GSK3β in multiprotein complex. Also moderate change was observed in the phosphorylation of GSK3β. It is suggested that lupeol interacts directly with GSK3β by enfolding to the ATP binding pocket of GSK3β (Figure 5) and inhibiting GSK3 mediated phophorylation of β-catenin (Harish *et al.,* 2008). This aspect seems to be responsible also for the wound healing activity of lupeol based on the signaling cascade of Wnt/β-catenin.

Figure 5: *The lupeol molecule enfolded in the ATP binding pocket of GSK 3-b showing interacting amino acids: Val 61, Ile 62, Asn64, Gly65, Ser66, Phe67, Gly68, Val 70, Lys 85, Leu*
132, Val 135, Pro 136, Asp181, Asp 20

1.6 GSKs in plants

Contrary to mammalian GSK3β, in plants little is known. Research in that field is just emerging and the first indications show involvement of GSK3β in different processes such as flower development, brassinosteroid signaling, NaCl stress and wound responses. Plant GSKs are encoded by a multigene family and in contrast to the highly conserved kinase domains, the N- and C-terminal regions of the plant GSKs differ considerably, suggesting that the various GSKs are involved in different biological processes. Based on protein sequence homology, the plant GSKs can be grouped into four classes (I–IV). In *Arabidopsis* there are ten different genes (Figure

6) encoding the GSK homologues which are termed as *At*SK or ASK in reference to the *Drosophila* GSK3 homologue (Saidi, Hearn and Coates *et al.,* 2012; Jonak and Hirt *et al.,* 2002).

Arabidopsis GSK3 clade	*Arabidopsis* GSK3	Gene identifier	Function/remark
I	*At*SK11/ASKα	At5g26750	Flower development/brassinosteroid signalling
	*At*SK12/ASKγ	At3g05840	Flower development/brassinosteroid signalling
	*At*SK13/ASKε	At5g14640	Osmotic stress induced/brassinosteroid signalling
II	*At*SK21/ASKη/BIN2/UCU1	At4g18710	Brassinosteroid signalling
	*At*SK22/ASKι/BIL1/AtGSK1	At1g06390	Brassinosteroid signalling/salt stress
	*At*SK23/ASKζ/BIL2	At2g30980	Brassinosteroid signalling
III	*At*SK31/ASKθ	At4g00720	Brassinosteroid signalling/osmotic stress induced
	*At*SK32/ASKβ	At3g61160	Flower development
IV	*At*SK41/ASKκ/AtK-1	At1g09840	Unknown
	*At*SK42/ASKδ	At1g57870	Osmotic stress induced

Figure 6: *Phylogenetic analysis of the ten Arabidopsis glycogen synthase kinase 3/SHAGGY-like protein kinases (GSKs). The Arabidopsis GSKs can be classified into four subgroups.*

Plant GSKs are molecules modulating various processes, important for the preservation of the plant such as hormone signaling, development and stress responses. There are scientific data that support the fact that GSKs play an important role during abiotic stress responses such as salt, drought or darkness (Piao *et al.,* 1999; 2001). GSKs are also important when it comes to biotic stress responses. Wounding is one of the most severe environmental stresses to which plants can be subjected and may be caused by mechanical injury, pathogen, or herbivore attack. Plants react to wounding by inducing defense responses characterized by the expression of a particular set of genes aimed primarily at preventing invasion by pathogens. Elicitors of plant defence cause result in activity change of kinases in different plants (Jonak *et al.,* 2000).

1.6.1 ASKθ and ASKη in brassinosteroid signaling

Brassinosteroids (BRs) are a class of steroid hormones essential for normal growth and development in plants. BR signaling involves the cell-surface receptor BRI1, the glycogen synthase kinase-3-like kinase BIN2 (ASKη) as a negative regulator, and the nuclear proteins BZR1 and BZR2/BES1 as positive regulators. BIN2 encodes a GSK3

with a catalytic domain sharing approximately 70% identity with animal GSK3β. Dominant mutations in BIN2 result in brassinosteroid-insensitive dwarf plants. In the absence of a BR signal, active BIN2 negatively regulates BR-specific transcription factors, BZR1 and BES1/BZR2 (Figure 7). Phosphorylation by BIN2 results in proteasomal degradation of BZR1 and BES1/BZR2. The function of BIN2 is consistent with mammalian GSK3. BIN2 induces the degradation and further promotes the nuclear export of BZR1/BES1, while GSK3 promotes the nuclear export of NFATc and StatA (Rybel *et al.,* 2009; Saidi, Hearn and Coates *et al.,* 2012; Yan *et al.,* 2009).

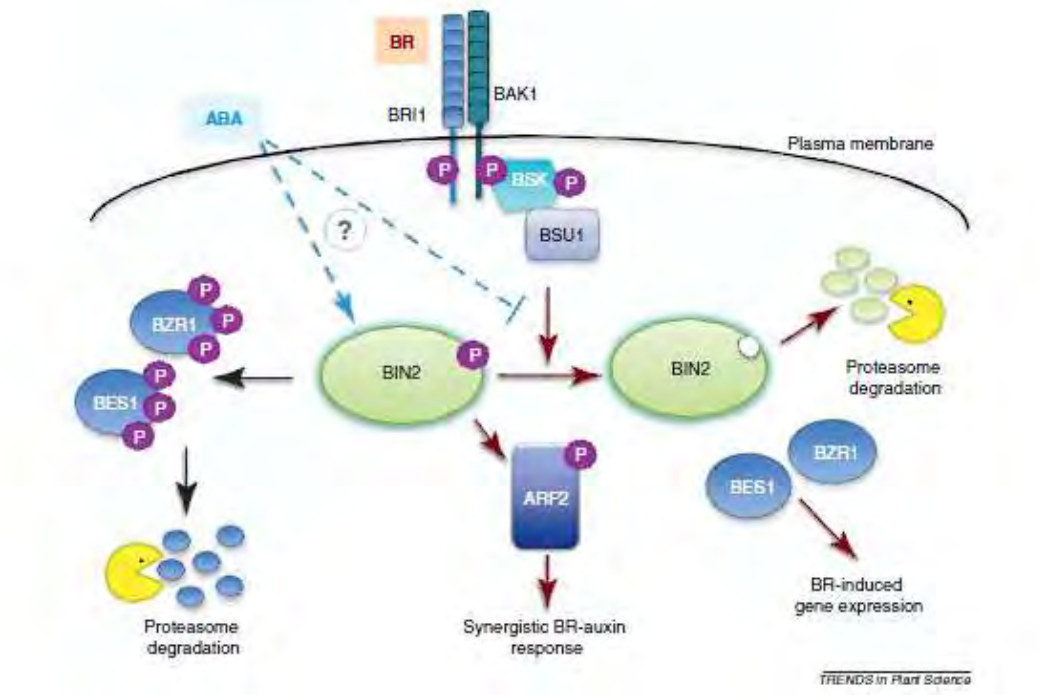


Figure 7: *The central role of BIN2 in BR signalling and its interface with other hormone pathways.*

In presence of BR signal the BRI1 receptor and its co-receptor BAK1 are activated. Transphosphorylated BRI1 phorsporylates BSK kinase which then activates BSU1 phospathase. BSU1 dephopshorylates BIN2, which is degraded and unphopshorylated BES1/BZR1 translocates to nucleus and modulates BR gene expression. BIN2 also phosphorylates the transcription repressor ARF2 and leads to a synergistic BR-auxin response. ABA inhibits BR signaling and enhances the

phosphorylated state of BES1. Except for ASKη (BIN2) two more kinases, ASKζ and ASKι, seem to be involved in BR signaling. In an effort to determine whether more shaggy like kinases are implicated, one group III-GSK was identified, ASKθ (Rozhon *et al.,* 2010). Several evidence shows that ASKθ is a negative regulator of BR signaling in a dose dependent manner. Moreover, ASKθ is believed to function downstream of the BRI1 receptor complex, and is negatively regulated by BRI1-dependent BR-stimulated signaling. It seems that ASKθ, BIN2, ASKι and ASKζ have overlapping but distinct expression patterns (Dornelas et al., 1999), and ASKθ and BIN2 displayed different binding specificities towards the distinct members of the family of BES1/BZR1- like transcription factors in yeast interaction assays pointing towards specific functions of ASKθ, BIN2, ASKι and ASKζ.

1.6.2 ASKα and abiotic stress response

During high salinity conditions an increase in reactive ogygen species can be observed and can result in oxidative damage. High salinity activates ASKα, which in turn phosphorylates G6PD6 on Thr-467, thus stimulating its activity. This was proved through quantification of G6PD6 activity in protoplast cells transformed with G6PD6 in presence and absence of ASKα. Enhanced G6PD activity provides enough NADPH for the antioxidant system in order to remove excess ROS. Reduction of H_2O_2 to water can then be mediated by the glutathione peroxidase cycle or by the ascorbate-glutathione cycle (Dal Santo *et al.,* 2012). ASKα was also reported to involve in flower meristem formation (Dornelas et al., 2000).

1.6.3 The role of GSKs in wounding

In alfalfa a member of the GSK3 family was rapidly induced during wounding. *WIG* (Wound Induced Gene) antibody studies revealed that despite transcript accumulation post trauma, protein levels remained stable and kinase activity was increased. Further inhibitor studies were conducted revealing that activation of WIG is modulated at a post-translational level. Also it seems that WIG inactivation is mediated from another protein kinase produced *de novo* after the wounding. SAMK kinase from MAPK pathway is considered as a candidate since in some mammalian

systems MAPK it is a negative upstream indirect regulator (Stambolic and Woodgett *et al.,* 1994; Jonak *et al.,* 2000).

1.7 GSKs in Lotus japonicus

Lotus japonicus belongs to the Leguminosae (Fabaceae) family, which is the third bigger family in the Angiosperms. It is a diploid, autogamous species, with relatively small genome (around 450 MB), and a generation time of approximately 3 months. What makes *L. japonicus* important for researchers is its ability to interact with nitrogen-fixing bacteria, form nodules and establish a symbiotic relationship. The symbiotic bacteria belong to *Mesorhizobium loti* strain. *M. loti* was isolated from the rhizosphere of *Lotus japonicus* in areas of New Zealand and China. It is a Gram-negative bacterium with mobility but it does not have the ability to fix nitrogen without a host-plant (Handberg and Stougaard et al., 1992). Nitrogen-fixing symbiosis between legumes and rhizobia is initiated by the recognition of rhizobial Nod factors (NFs) by host plants. NFs are diversely modified derivatives of chitin oligosaccharide, a fungal elicitor that induces defense and symbiotic responses in plants. The production of NFs is activated from phenolic substances mainly flavonoids which are released from the plant towards the rhizosphere. Nod factor receptor is a heterodimer of two kinases each of them carrying a LysM domain on the extracellular area. LysM domains bind oligosaccharides that constitute the backbone of Nod factors. Mutations in these receptors inhibit every course of action in response to NFs (Nakagawa et *al.,* 2010). This receptor-like kinase mediated pathway share the early responses with defense against pathogens pathway which is activated by elicitors such as flagellin (flg22) (Antolin-Llovera *et al.,* 2012). However the downstream elements in both pathways are not yet fully described and characterized.

In *Lotus japonicus* little is known about kinases that implicate in signaling pathways. According to Kameshita *et al.,* 2004, 164 cDNA clones were found to encode putative Ser/Thr kinases or kinase-like proteins, and these cDNAs were attributed to 15

different genes that have not been reported previously. A phylogenetic analysis that followed, classified the putative kinases into five different families of Ser/Thr kinases, (i) Ndr family, (ii) SnRK family, (iii) RLK family, (iv) ARK family and (v) GSK3 family (Figure 8). GSK3 family had two members LNZ020 and LNZ031.

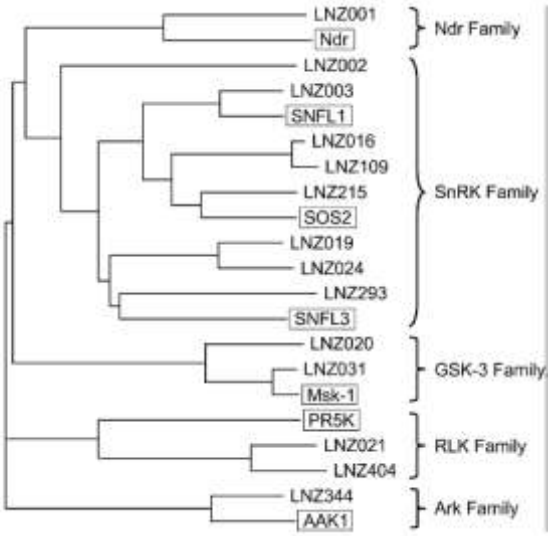


Figure 8: *Phylogenetic analysis of putative Ser/Thr kinases.*

2. Aim of the study

In mammals, the characterization of Glycogen Synthase kinases has attracted significant research efforts. The same does not apply in plants where very little is known to date. The most extensively studied plant GSKs are those belonging to the model-plant *Arabidopsis thaliana*. The purpose of this study is to isolate and study GSK genes in *Lotus japonicus*. The research on these genes was mainly focus on the aspects concerning the implications of GSKs in legume-rhizobium symbiosis as well as on the interaction and modulation of the kinase activity by natural compounds known as terpenes, specifically lupeol.

3. Materials and Methods

3.1 Agrobacterium rhizogene-mediated root transformation

1. Preparation of the plasmid construct for the production of siRNA

- Amplification of a 257bp gene fragment from *Lj*GSK3β using the primers below, designed for cloning in the pENTR4 vector:
  pENTR4 Forward with integrated restriction site for NcoI
  GACCCATGGATGAACATGATGAGACGG

  pENTR4 Reverse with integrated restriction site for XhoI
  AGCGCTCGAGCTCTTTTGGAAGTTCATC

  The PCR reaction was performed with Phusion polymerase from Finnzymes

	Volume	Final Concentration
Template DNA	1μl	10ng
Primer 1	5μl	0,5μM
Primer 2	5μl	0,5μM
dNTP's mixture	1μl	200μM each
reaction buffer 5x	10μl	1X
Phusion	0,5μl	0,02 unit/μl
ddH_2O	up to 50μl	-

The program for the amplification was:

Amplification conditions	
Initial denaturation	98^0C for 30 seconds
Denaturation Primer annealing Elongation	98^0C for 5 seconds 72^0C for 15 seconds 72^0C for 15 seconds number of cycles performed 30
Final denaturation	72^0C for 5 minutes

- PCR clean up using the PCR cleanup kit from Macherey-Nagel, according to the manufacturer's instructions
- Double digestion of both the PCR fragment and the vector, using the enzymes NcoI HF and XhoI from New England Biolabs at 37^0C for 3-4 hours with buffer 4 and BSA. Digestion was followed by agarose gel electrophoresis (1,5% agarose) and gel extraction using the gel extraction kit from Macherey-Nagel according to the manufacturer's instructions
- Ligation reaction was performed using Takara ligase. The digested PCR fragment and pENTR4 vector were incubated in the ligation mixture for 16 hours at 4^0C.
- DH5a competent cells were transformed with the ligation mixture.
- The occurring colonies were screened for carrying the insertion using colony PCR and digestion with restriction enzymes.
- Plasmid was purifiedfrom the positive colonies using Plasmid prep kit from Macherey-Nagel, according to the manufacturer's instructions
- The construct was inserted into the final vector (pUbi-GWS-GFP), using a Gateway cloning strategy. LR clonase from Life Sciences was used, and the reaction was based on the following protocol:

  Destination vector pUbi 1µl (150ng)

Pentr4-GSK3 plasmid 1μl (150ng)

LR clonase 2μl

TE buffer pH8 6μl (up to 10μl)

The above mixture was incubated for 16 hours at 25^0C and 1μl of proteinase K was then added, followed by incubation for 10 minutes in 37^0C in order to stop the reaction. Transformation of DH5a cells with the ligation mixture was performed and the positive colonies were detected by colony PCR using primers amplifying either the GSK3β fragment or the intron localized on the destination vector.

The plasmid containing the full construct was extracted from the positive E. coli colonies and approximately 200 ng were used to transform LBA1334 *Agrobacterium rhizogenes* cells. Again the occurring colonies were tested by PCR to ensure the presence of the insert in them.

2. Seed sterilization

- Approximately 300 Lotus japonicus GIFU seeds were divided into eppendorfs containing roughly 80 seeds per tube. Into each eppe*ndorf 1ml* solution was added, containing 750μl ddH_2O, 250μl commercial bleach and 1μl Triton X100.
- After gently shaking the eppendorfs for 20 minutes, sterile ddH_2O was added six times in order to wash the sterilization solution.
- After the last washing, the seeds were left into 1ml ddH_2O, at 4^0C overnight.
- On the next day, the seeds were lined onto Petri dishes containing 1% water-agar medium. The dishes were covered by aluminium foil and left at 4^0C overnight.
- Finally the Petri dishes containing the seeds are transferred the next day (still covered) in the growth chamber, incubated for 3 days and the cover was removed in order to adjust to the light/dark cycle. The conditions were 23^0C, 16 hours light/8 hours dark

3. Infection of *L. japonicus* by *A.rhizogenes*

- Transformed *Agrobacterium rhizogenes* cells were plated in LB plates with the appropriate antibiotics (in our case spectinomycin 250μg/ml, rifampicin 50μg/ml, kanamycin 50μg/ml) and grew in 28^0C for 3 days in the dark.
- Single colonies were then transferred in 5 ml of liquid LB medium containing the same antibiotics and grew for 2 days at 28^0C.
- When the liquid cultures had grown, 500μl were plated in LB plates antibiotics and grew overnight at 28^0C.
- The following day, the root of the plants was removed, they were infected with the *A. rhizogenes* liquid culture, and then transferred into square dishes containing B5 medium, 2% sucrose and 1% agar and left horizontally in the dark in growth chamber.
- Two days after the infection, the plants were exposed to normal light/dark cycle.
- Three days later plants were washed, in case there was an immense growth of *Agrobacterium*, and then plated in plates containing B5medium, 2% sucrose, 1% agar and 300μg/ml cefotaxime. The Petri dishes were kept vertically in the growth champer.
- One week later, plants were transferred into Petri dishes containing Jensen medium with 1% agar. At this point roots grown above the infection site were removed.
- 10 days later, the transformed roots were screened for expression of GFP using a fluorescence microscope. The destination vector (pUbi) carry the GFP gene allowing the detection of transformed roots by fluorescence.
- Two days after the screening, the plants carrying transformed roots were divided into subgroups and half of them were inoculated with *M.loti* liquid culture OD_{600} 0,1 .
- Roots of both inoculated and non-inoculated plants were harvested 1 hour after the inoculation and flash-frozen in liquid nitrogen.

4. RNA extraction, DNase treatment and cDNA synthesis

- RNA was extracted from the roots using RNA extraction kit from Qiagen, according to the manufacturer's instructions

- quantity and quality of the RNA were determined spectrophotometrically by measuring the absorbance at 260 nm and by electrophoresis on 2% w/v agarose gel.
- The RNA samples were treated with DNase I from Invitrogen. The samples were incubated in 37^0C for 1 hour before the inactivation of the DNase at 65^0C for 10 min. In order to ensure no genomic DNA was left, a PCR was performed using primers specifically designed to amplify the housekeeping gene *UBIQUITIN* of *Lotus japonicus*.
- cDNA synthesis was performed using Reverse Transcriptase from Invitrogen

Quantitive Real Time Polymerase Chain Reaction was performed for the normalization of the cDNAs and for the calculation of the expression level of *LjGSK3β*. Quantitative RT-PCR reactions were performed on the Stratagene MX3005P using SYBR Green mix (Kapa), gene-specific primers at a final concentration of 0.2 μM each and 1 μl of the cDNA as template. PCR cycling started with the initial polymerase activation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 11 s. The primer specificity and the formation of primer-dimmers were monitored by dissociation curve analysis. The expression levels of a *L. japonicus* ubiquitin gene were used as internal standards to normalize small differences in cDNA template amounts. Relative transcript levels of the gene of interest (X) were calculated as a ratio to the ubiquitin gene transcripts (U), as $(1+E)^{-\Delta Ct}$, where ΔC_t was calculated as $(C_t^X - C_t^U)$. PCR efficiency (E) for each amplicon was calculated employing the linear regression method on the Log (Fluorescence) per cycle number data, using the LinRegPCR software. All real-time qPCR reactions were performed on 3 biological repeats. The primers used were:

GSK3bF_RT	5'-CCTATTGGTTAATCCCCAGACA-3'
GSK3bR_RT	5'-ACAACCAACAGACCACATATCG-3'

3.2 Gene expression after external application of terpenes in plants

1.Plant material and growth conditions

- *Lotus japonicus* (*cv* Gifu) seeds were sterilized for 20 min in NaOCl and rinsed with sterile distilled water six times. The seeds were left in water filled eppendorfs overnight at 4^{o} C in the dark. They were then pregerminated in Petri dishes containing 1% water-agar at 4^{o}C in the dark for 1 day and at 22^{o}C for 2 days under a 16 h: 8 h light : dark cycle.

2.Experimental procedure

- The seedlings were transferred in Petri dishes containing Jensen medium and each root was inoculated with 15μl *M.loti* (strain R7A) OD_{600} 0,1 suspension culture. The inoculated plants were divided into treatment groups, in which 15μl of 100μM lupeol or β-amyrin or betulinic acid were added per plant. The roots were harvested at 15min and 48 hours after plant inoculation with *M.loti*. Control plants were infected with *M.loti* and treated with the solvent minus the terpenes. Uninfected plants both treated and untreated with terpenes were also used as controls within the treatment groups.

3.Real-time PCR experiments

- Roots at 15min and 48 hours were harvested and flash-frozen in liquid nitrogen. Total RNA was isolated from approximately 9 roots per treatment group with each terpene for infected and control plants using the Qiagen RNeasy Mini Kit, according to the manufacturer's instructions. Total RNA concentration was quantified spectrophotometrically by measuring the absorbance at 260 nm and by electrophoresis on 2% w/v agarose gel. To eliminate residues of DNA, the samples were treated with DNase I by Invitrogen at 37^{o}C for 1 hour and primers amplifying the housekeeping gene *UBIQUITIN* (*UBQ*) were used in PCR to test the complete removal of DNA.

- First strand cDNA was reverse transcribed with SuperScript II Reverse Transcriptase by Invitrogen. The resulting cDNA was normalized based on the expression of *UBQ*. Quantitative reverse transcription-polymerase chain reaction was performed for *LjGSK3-β* gene. The expression levels of *L. japonicus* UBQ gene were used as internal standards. Relative transcript levels in different samples for the gene of interest were calculated as a ratio to the *UBQ* gene transcripts.

3.3 Autophosphorylation assay

The GSK3 auto-phosphorylation was tested by a biochemical assay. This assay is based on a series of different reactions linked together and having as a result a decrease in absorbance of a specific component. More specifically it is based on coupling the ADP production from the phosphorylation capacity of a certain kinase to the NADH oxidation by pyruvate kinase and lactate dehydrogenase. The NADH oxidation leads to a decreased absorbance at 340nm.

Experimental Procedure

In order to evaluate the autophosphorylation activity of *Lj*GSK3β three different test tubes were prepared. The first one contained a mixture of the buffer and the enzymes, pyruvate kinase and lactate dehydrogenase. In the second tube ATP was also added to the previous mixture to measure any ATPase activity for which these enzymes may be responsible. In the third tube, both ATP and *Lj*GSK3β were added to the previously stated mixture. For the assays performed in presence of lupeol, either lupeol or the solvent of the terpene, DMSO, was added to the above mentioned mixtures.

Components	Stock solution	Final concentration
Phosphoenolpyruvic acid	0,1M	1mM
NADH	0,01M	0,125mM
BSA	10mg/ml	0,5mg/ml
NaCl	1M	100mM
MgCl2	1M	10Mm
DTT	0,1M	1mM
ATP	100Mm	1mM
Tris-HCl	1M	20mM
Lactate dehydrogenase	243,38u/ml	30u/ml
Pyruvate kinase	3953,6 u/ml	12u/ml
***Lj*GSK3β**	3,8mg/ml	1μg/ml
Lupeol	50mM	10μM
DMSO	100%	quantity that equals the content in the lupeol final solution

All reactions were performed in a final volume of 800μl. Every component of the solution was added from the beginning of the assay except from ATP and *Lj*GSK3β. After the OD_{340} was stabilized the kinase and the ATP were added (the ATP was always the last one added). The solutions were then mixed with light pipeting. Reactions were stopped when the OD reached a very low level compared to the beginning.

Autophosphorylation assay:

No ATP

No *Lj*GSK3β

Plus ATP

No *Lj*GSK3β

Plus ATP

Plus *Lj*GSK3β

Autophosphorylation assay with lupeol/DMSO:

No ATP

No *Lj*GSK3β

Lupeol

Plus ATP

No *Lj*GSK3β

Lupeol

Plus ATP

Plus *Lj*GSK3β

Lupeol

No ATP

No *Lj*GSK3β

DMSO

Plus ATP

No *Lj*GSK3β

DMSO

Plus ATP

Plus *Lj*GSK3β

DMSO

3.4 Protein expression and purification

A. Expression in pET28a

1. Insertion of *Lj*GSK3β in pET28a expression vector

- The full length *Lj*GSK3β and the pET28a vector were digested using EcoRI and XhoI enzymes at 37^0C for 4 hours, followed by gel extraction with Gel extraction and PCR clean up kit from Macherey-Nagel.
- Insert and vector were ligated using Takara ligase. The quantity of vector was estimated at 100ng and of the insert using the formula:

$$\frac{\text{ng of vector x kb size of insert x}\frac{\text{insert}}{\text{vector}}\text{ molar ratio}}{\text{kb size of vector}}$$

- DH5a cells were transformed with the ligation mixture. Plasmid was extracted from liquid cultures of selected growing colonies using Plasmid prep kit from Macherey-Nagel and diagnostic digestions confirmed the presence of *LjGSK3β* insertion in the *E. coli* colonies.
- BL21 and Rosetta cells were subsequently transformed with the constructed plasmid for protein expression. The expression system based on Rosetta cells appeared more suitable for our protein.

2. Protein expression and cell lysis

- Streaking in a petri dish with LB-kanamycin medium, overnight incubation at 37^0C.
- Single colony was transferred in 5ml of LB-kanamycin medium and incubated at 37^0C, 210rpm for 12-16 hours.
- 2,5 ml from the overnight culture transferred in 50ml LB with antibiotics (1/20 final dilution), grown at 37^0C, 210rpm until the OD_{600} reaches 0,5-0,7

- A sample of 1ml from the culture kept as a noninduced control . The cells were pelleted with centrifugation at maximum speed for 1 minute. Pellet was kept at -20^{0}C until needed for SDS-PAGE.
- Expression induced by IPTG in a concentration range of 0,1mM-1mM, in different temperatures (18-37^{0}C) for 4 hours. An 1 ml sample of the culture was kept as induced control.
- The cells were harvested be centrifugation at 4000 rpm for 20min and the pellet was kept at -20^{0}C.
- Cell pellet resuspended in 5ml of lysis buffer:

  NaH_2PO_4 50mM
  NaCl 300mM
  Imidazole 250m pH 7,5

- Lysozyme was added to a final concentration of 1mg/ml and the lysate was incubated on ice for 30min
- Sonication followed for 6 times x 10sec with 10sec pauses. Lysate was kept on ice at all times
- The lysate was centrifuged at 8000rpm at 4^{0}C for 20 min. The supernatant contain the soluble proteins.
- The pellet is resuspended in Tris-Urea 8M. This suspension contain the insoluble proteins.

3. SDS-PAGE electrophoresis

- Preparation of SDS-PAGE gel. The separating gel that was used had 12% polyacrylamide content

  For preparation of 10ml separating gel:

Components	Volume
H20	3,3ml
30% acrylamide mix	4ml
1,5M Tris-HCl pH 8,9	2,5ml
10% SDS	0,1ml
TEMED	10µl
10% APS	0,1ml

For preparation of 5ml stacking gel:

Components	Volume
H20	3,4ml
30% acrylamide mix	0,630ml
1M Tris-HCl pH 6,8	0,630ml
10% SDS	0,05ml
TEMED	5µl
10% APS	0,05ml

- Buffers and procedure for staining and destaining the SDS-PAGE gel:

Coomasie staining solution 1lt:

H_2O 450ml

Methanol 450ml

Acetic acid 100ml

Coomasie brilliant blue R250 0,25 gr

The gel was incubated in room temperature in the staining solution until stained or overnight

Destaining solution and protocol:

Destain solution 1: 30% methanol
10% acetic acid ⟹
- Leave in the destain 1 for 1 hour
- Decant the old destain 1 and add a new, leave for another hour

Destain solution 2: 5% methanol
7% acetic acid ⟹
- Preserve in destain solution 2

4. Protein purification using Ni-NTA Agarose beads

- A 600ml bacterial culture was grown at 37^0C until the OD_{600} reached 0,6 and the protein expression was induced by 0,5mM IPTG for 4 hours. The cells were pelleted at 8000rpm, for 5min at 4^0Cand the bacterial pellet was kept at either -20 or -80^0C.
- Pellet resuspended in final volume of 40ml lysis buffer, with 0,1% Triton X and 0,5 mM PMSF, followed by vortexing.

  Lysis Buffer (buffer A): Tris 50mM
  NaCl 100mM pH 7,5
  10% glycerol
- The lysate was sonicated for 15min 2 times with 15min pauses at 30% amplification. Falcon tubes always kept on ice.
- Transfer the supernatant in new falcon and add 300μl beads (Protino Ni-Nta Agarose from Macherey-Nagel)
- The falcon containing the supernatant and the beads is left on a turning wheel at 4^0C for 2 hours.
- Centrifuge for 3min, 3000rpm at 4^0C
- Decant the supernant carefully in order to keep the beads intact
- Wash the beads by adding 15ml of buffer A and centrifuging. Repeat two times

- At the last wash resuspend the beads in 500μl buffer A and transfer the solution in a mini plastic column
- Wash three times with 500μl buffer A
- Wash once with 500μl buffer A plus 0,2% Triton
- Wash two times with 500μl buffer A
- Add 200μl buffer A plus DTT (DTT 1M→10μl for 1ml buffer)
- Close the column, add 200μl imidazole 0,5M and incubate 30 min on the spinning wheel at 4^0C. Collect the eluate
- Add again 200μl imidazole to the column and incubate on the spinning wheel at 4^0C overnight.
- Gather the second eluate and wash the beads either with H_2O or with buffer A
- The eluates are analysed on an SDS-PAGE gel

B. Expression in pGEX-6P-1 vector

1. Insertion of *Lj*GSK3β in pGEX-6P-1 expression vector

- The full length *Lj*GSK3β and the pGEx vector were digested using EcoRI HF and XhoI enzymes at 37^0C for 4 hours, followed by gel extraction with Gel extraction and PCR clean up kit from Macherey-Nagel.
- Insert and vector were ligated using Takara ligase. Quantity of vector was estimated at 100ng and of the insert using the formula:

$$\frac{\text{ng of vector x kb size of insert x}\frac{\text{insert}}{\text{vector}}\text{ molar ratio}}{\text{kb size of vector}}$$

- DH5a cells were transformed with the ligation mixture. Plasmid from liquid cultures was extracted using Plasmid prep kit from Macherey-Nagel and diagnostic digestions confirmed the correct insertion of *LjGSK3β*.
- BL21 and Rosetta cells were used for protein expression. The expression system based on Rosetta cells appeared more suitable for our protein.

2. Protein expression and cell lysis

- Dilute an overnight culture 1/10 in fresh LB-ampicillin medium. Leave the cells to grow until OD_{600} is 0,6 at 37^0C, 210rpm
- 1ml from the culture is kept as a noninduced control with cells pelleted with centrifugation at maximum speed for 1 minute. Pellet is kept at -20^0C oC until needed for SDS-PAGE.
- Expression induced using IPTG in a concentration range of 0,1mM-1mM, in different temperatures (18-37^0C) for 4 hours. A 1 ml sample of the culture was kept as induced control.
- The cells are harvested be centrifugation at 4000 rpm for 20min and kept at -20^0C
- Cell pellet resuspended in PBS buffer: 140 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 1,8 mM KH_2PO_4, pH 7.3
- Sonication followed for 6 times x 10sec with 10sec pauses. Lysate was kept on ice at all times, then Triton X was added to a final 1% concentration and the lysate was kept on ice for 30min in order to solubilize the proteins.
- The lysate was then centrifuged at 8000rpm at 4^0C for 20 min. The supernatant may contain the soluble protein.
- The pellet is resuspended in PBS buffer or Tris-Urea 8M. This suspension will contain the insoluble protein.

3. Protein purification with GST beads

- After the expression was induced for 4 hours in final volume of 600ml (37^0C and 0,2Mm IPTG were the preferred conditions at this point), cells were pelleted at 8000rpm, for 5min at 4^0C (max 2 centrifugations/falcon). Cell pellet kept at either -20 or -80^0C.
- Pellet resuspended in final volume of 40ml lysis buffer, with 0,1% Triton X and 0,5 mM PMSF, followed by vortexing.

  Lysis Buffer: Tris 50mM
  NaCl 100mM pH 7,5
  10% glycerol

- Sonicate the lysate for 15min 2 times with 15min pauses at 30% amplification. Falcon tubes always kept on ice.
- Transfer the supernatant in new falcon and add 300µl beads The falcon containing the supernatant and the beads is left on a turning wheel at 4^0C for 2 hours.
- Centrifuge for 3min, 3000rpm at 4^0C
- Decant the supernant carefully in order to keep the beads intact
- Wash the beads by adding 15ml of Lysis buffer and centrifuging. Repeat two times
- Resuspend the beads in 1ml lysis buffer and transfer the solution in the column
- Wash three times with 500µl lysis buffer
- Add carefully on the sides of the column 500µl lysis buffer and 0,2% Triton
- Wash two times with 500µl lysis buffer
- Add 200µl lysis buffer and DTT (DTT 1M→10µl for 1ml buffer)
- Add 200µl lysis buffer with the appropriate concentration on 3C-GST protease. Incubate on the spinning wheel overnight at 4^0C
- Gather the solution and wash the beads by adding lysis buffer. Store beads in 4^0C in case there is protein left on them and further elution is needed.

4. Protein purification with FPLC

The construct used for the expression and purification of LjGSK3 protein by FPLC was the LjGSK3-pGEX due to higher expression efficiency.

Protein expression and cell lysis

Protein was expressed and induced at 37 ^{o}C for 4 hours with 0,2mM IPTG. Total volume of the culture was 3,2lt. Cells were pelleted by centrifugation at 5000rpm for 10min.
Cell pellet was preserved in -80 ^{o}C until lysis.

Lysis Buffer
50Mm KH2PO4 pH 7,5

0,2M NaCl

0,1% MTG

Protease inhibitor mix Roche

Total volume 50ml, filter sterilized

5-6ml of lysis buffer were added to one of the pellets and it was then dissolved by pipetting. The same 5-6 ml with the dissolved pellet were transferred to the next falcon until the next pellet was also dissolved etc. Maximun volume of dissolved pellets should not exceed 20-30ml. Addition of benzonase (1μl of 5ku to a final 30ml sample volume) followed and the sample was left on ice for 5min. Cell lysis was achieved by sonication for 30sec, 6 times with 30 sec pause intervals, at 60-70% amplification, 1 cycle. To separate the soluble and insoluble parts, the sample was centrifuged at 11000 rpm for 45min.

First column-GST binding column

- Purification with FPLC was executed according to instructions provided by the manufacturer.

  Buffers used:

  Buffer A
  50Mm KH_2PO_4
  0,2M NaCl
  10% glycerol pH 7,5
  0,1% MTG

  Buffer B
  50Mm KH_2PO_4
  0,2M NaCl
  10% glycerol pH 7,5
  20mM Glutathion reduced
  Column equilibration buffers:

1. 100mM Tris pH 8,5
   0,5M NaCl

2. 10mM Sodium Acetate pH 4,5
   0,5M NaCl

After GST column, the GST tag protein from the elution sample was cleaved using 3C protease. For every 20mg of protein (estimated with Bradford) 1mg of 3C is required but since 3C was not commercial it needed twice as much.

Second column- Kinase specific cibacron

In order to pass the sample through cibacron, it was diluted 4 times due to the concentration of NaCl in the buffers. Buffer A from GST column has 200mM NaCl whereas Buffer A for cibacron has 50mM NaCl. For the dilution process the appropriate volume of KH_2PO_4, glycerol and H2O was added.

Buffer A cibacron:

50mM KH_2PO_4
50Mm NaCl pH 7,5
10% glycerol
0,1% MTG

Buffer B cibacron

50Mm KH_2PO_4
1M NaCl pH 7,5
10% glycerol
0,1% MTG

At this point, the sample is quite pure but it still has a small quantity of 3C, or a small cleaved GST-tag. To further cleane the protein we proceed to a **third column**, a GST binding column again. Buffers used were the same as the first column. This time our protein is in the flow through.

After protein is acquired and probably condensed using filter tubes, it is preserved in the following buffer in small volumes at -80^{o}C:

Preservation Buffer:
10mM HEPES pH 7,5
2% glycerol
2mM $MgCl_2$
1Mm DTT

*All buffers for the FPLC procedure were kept at 4^{0}C
All elutions were isocratic, gradient was not needed

After each column, all samples were analysed on SDS-PAGE gel and the gel was stained and destained the same day according to the following procedure:

- 10% Acetic acid was added to the gel and boiled in a microwave for a few minutes.
- The boiling acetic acid was discarded and gel went in a Coomasie dye filled vessel. Left to boil in a microwave and then incubated with 20rpm at room temperature for 10 minutes.

- After 10 minutes passed, Coomasie was returned to its original bottle until next use, and in the gel 10% acetic acid was added and boiled again in the microwave. Both addition of acetic acid and boiling were repeated two more times. The gel was then destained.

4. Results

4.1. Database screening for the proposed gene sequence

Initially based on the results by Kameshita *et al.,* 2004, we tried to locate a *GSK3β* gene sequence in *Lotus japonicus.* Using NCBI, two kinases (clone LNZ020, sequence ID: AB113573.1 and clone LNZ031, sequence ID: *Lj*T36I04) were found. The first kinase (Figure 1), AB113573.1, has a 76% identity to *Arabidopsis* ASK theta (ASKθ), a molecule playing an important part in brassinosteroid signaling (Rozhon *et al.,* 2010). The second shares an 89 % identity with AB113573.1 and is 78 % similar to another *Arabidopsis* kinase, ASKα (Dal Santo *et al.,* 2012) which is activated during redox stress response.

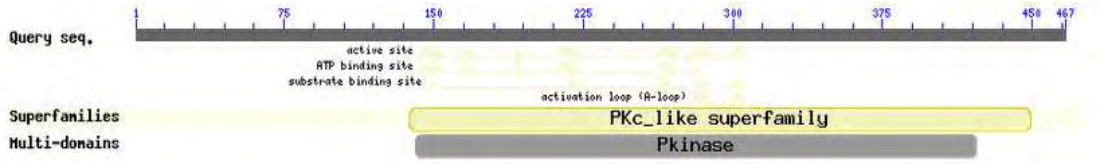


Figure 1: *Graphical summary of the conserved domains of LjGSK3β* (*AB113573.1*) *as shown in NCBI*.

4.2. Gene amplification using PCR and cloning

Full sequences were available for both kinase molecules. Our main target was the first kinase, the one resembling ASKθ, due to its putative role in pathways involved in symbiotic processes and hormone signaling and from this point on it will be referred as *LjGSK3β*. For the full sequence amplification the two primers described in Table 1 were used. As matrix for the polymerase chain reaction, cDNA from roots was used and the expected length was 1404 bp.

GSK.EcoRI_F	5'-ACGGAATTCATGAACATGATGAGACGG-3'
GSK.Xho_R	5'-AGCTCTCGAGTCAACTCCTTGCATGCTC-3'

Table 1: Primers for the amplification of *LjGSK3β*

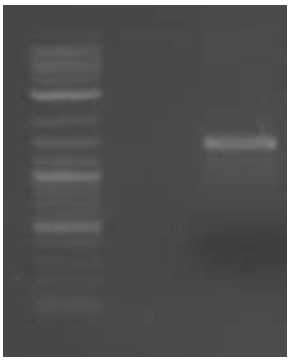


***Figure 2**. Agarose gel (1%) electrophoresis of the PCR results for the LjGSK3β.*

4.3. Expression analysis of *LjGSK3β* in *Lotus japonicus* root.

In order to analyze the expression of *LjGSK3β,* quantitative real time PCR was performed in *Lotus japonicus* roots. The cDNA was from 8 days old plants. On the eighth day half of the plants were inoculated with *M.loti* and plant tissue was collected at two different time points (1hour and 7 hours post inoculation) and frozen in liquid nitrogen. Plant tissue from both infected and uninfected roots was used for RNA extraction. The expression analysis showed a statistically significant difference in *Lj*GSK3β expression between tissues of infected with rhizobium and those uninfected plants (Figure 3) at 1 hour post inoculation. For the Quantitative Real Time Polymerase Chain Reaction the primers in Table 2 were designed.

GSK3bF_RT	5'-CCTATTGGTTAATCCCCAGACA-3'
GSK3bR_RT	5'-ACAACCAACAGACCACATATCG-3'

Table 2: Real Time Polymerase Chain Reaction primers for *Lj*GSK3β.

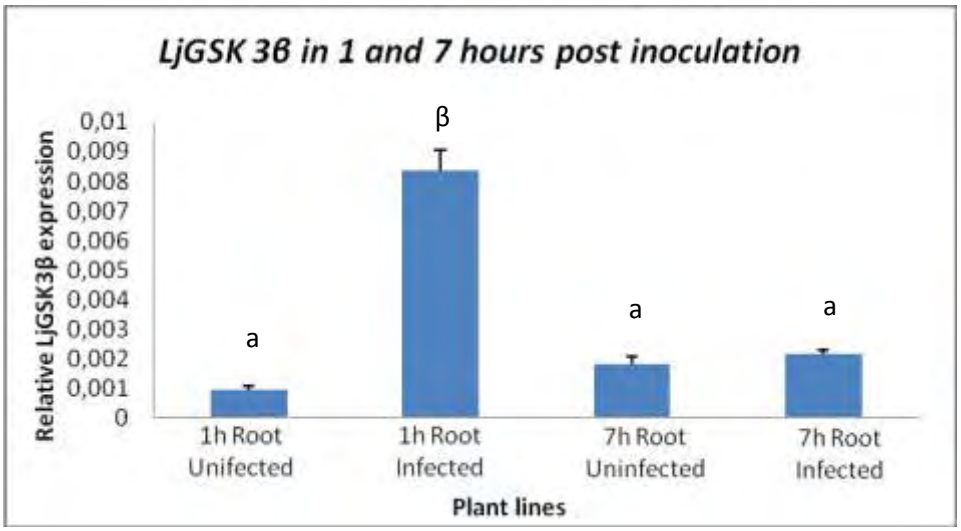


Figure 3. *Graph with the results of real time PCR. Expression of LjGSK3β during the first hour post inoculation is significantly elevated compared to the other time points.*

Furthermore, in order to verify the difference between infected and unifected plants another experiment was held. Plants of 8 days were infected with *M.loti* and again tissue from both infected and uninfected plants was collected at 1 hour post inoculation. RNA extraction followed and Real Time PCR was performed in cDNA from roots. LjGSK3β expression levels show a statistical significance between infected and uninfected plants. Specifically expression is almost two times higher in plants infected with rhizobium compared to plants not infected.

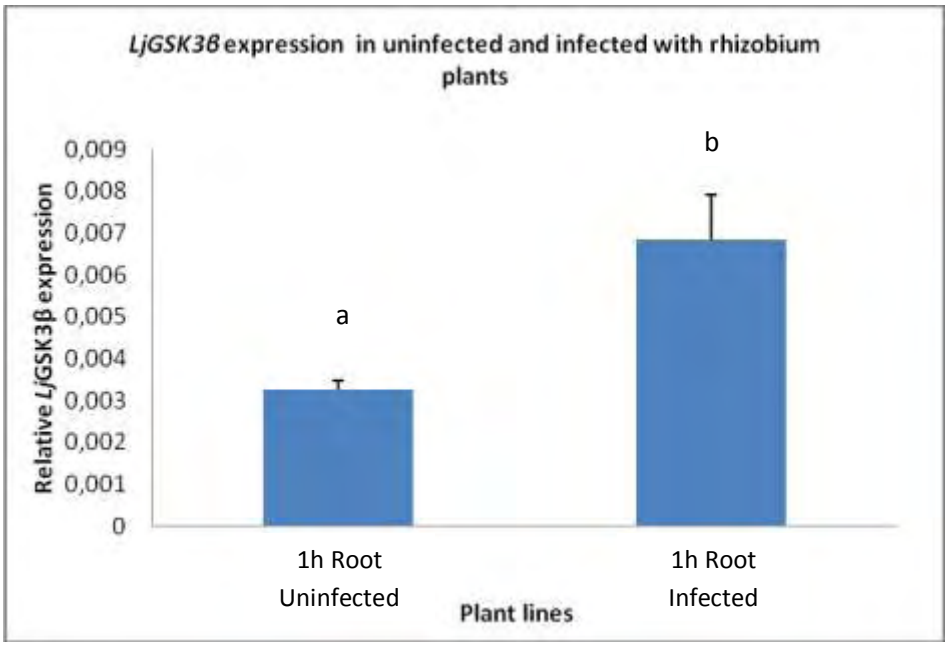


Figure 4. *Real time PCR graph that focuses between the infected and uninfected roots expression pattern of LjGSK3β.*

4.4. Agrobacterium rhizogenes mediated silencing of *LjGSK3β*

In order to investigate the role of *LjGSK3β* we proceeded in gene silencing using the siRNA technique provided by the intermediate vectors pENTR4 and pUBI-GWS-GFP. The last vector is used both for the final siRNA production and for the screening of successful transformation of *Lotus japonicus* using Green Fluorescence Protein. A fragment of *LjGSK3β* approximately 200bp was inserted in pENTR4 vector using restriction enzymes. The enzymes used were NcoI and XhoI and specific primers were designed (Table 3). The fragment was inserted between two sites that can be recognized by Clonase enzyme. Using this enzyme the fragments were transferred in pUBI vector. The two fragments in pUBI were expressed as siRNAS thus enabling the silencing of the gene.

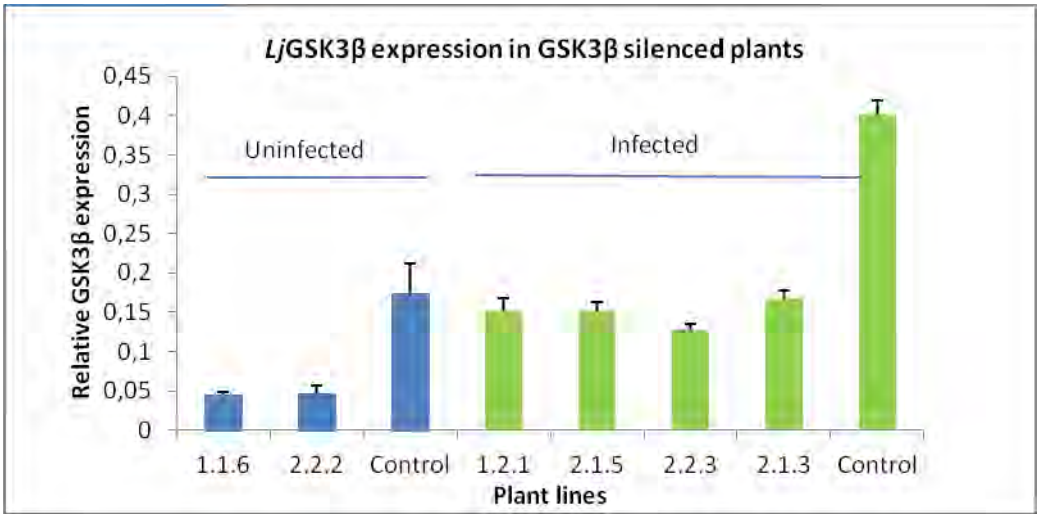
After the screening with GFP was over and the plants had developed their transformed roots, inoculation with *M.loti* followed. Plant tissue was collected one hour post inoculation. The cDNA used for the RNA extraction and real time PCR experiments was from roots in order to establish the exact percentage of *LjGSK3β* silencing in each plant (Figure 5). Plants showing the greater percentage of gene

silencing were chosen and used for more Real Time PCR experiments on different genes.

For further Real Time PCR experiments the genes were chosen based on their putative implication on the brassinosteroid and immune response pathway thought to be regulated by *LjGSK3β* with the exception of *Lj*T36I04 which was studied mainly due to the similarity to *LjGSK3β.* Indeed *Lj*T36I04 showed a significant reduction of expression levels(Figure 6) in the same plants that *LjGSK3β* was silenced. The only difference was the extreme silencing percentage in the uninfected plants compared to the infected. *Lj*T36I04 silencing occurred probably due to the highly similar sequences between the two genes which enabled the designed primers to anneal with areas on the *Lj*T36I04 gene. As for *Lj*PUB13 expression levels, they were significantly different between silenced and control plants but no difference in expression was observed between the infected plants. Also there was a significant difference in expression between the uninfected and infected control (Figure 7).

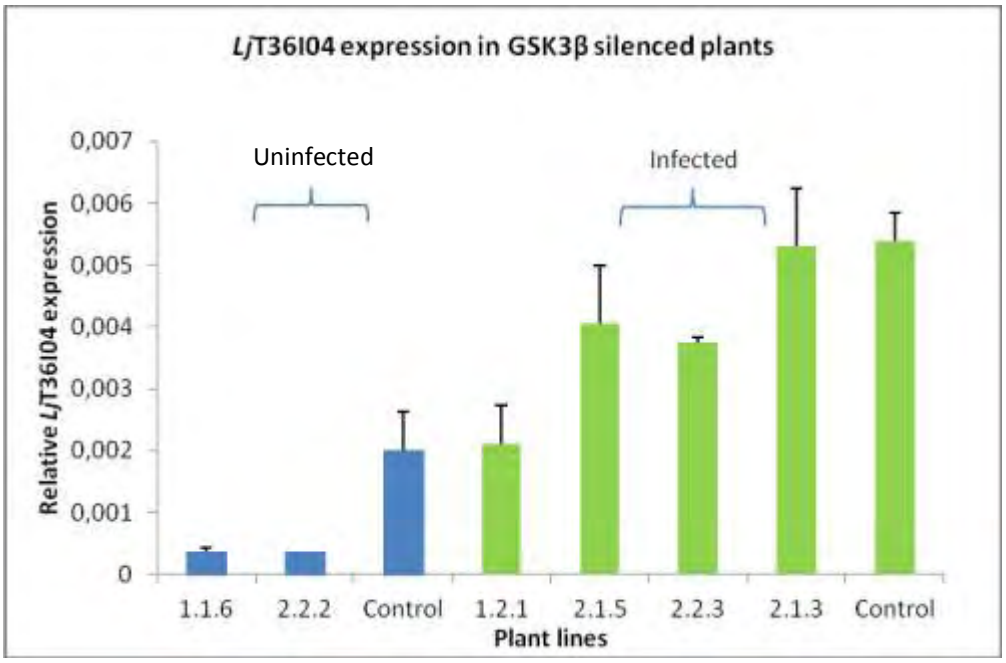
pENTR4-pUBI Forward NcoI	5'- GACCCATGGATGAACATGATGAGACGG- 3'
pENTR4-pUBI Reverse XhoI	5'- AGCGCTCGAGCTCTTTTGGAAGTTCATC- 3'

Table 3: Primers designed for *Lj*GSK3β silencing



Silencing level of *LjGSK3β*	Uninfected	Infected
	Plant 1.1.6 - 63,7%	Plant 1.2.1 - 41,3%
	Plant 2.2.2 - 62%	Plant 2.1.5 - 41,2%
	-	Plant 2.2.3 - 50%
	-	Plant 2.1.3 - 35%

Figure 5*. Real time PCR results for the LjGSK3β gene expression levels one hour post inoculation in selected transformed plants expressed as percentage reduction of control levels (mean value of X/Y for the uninfected/infected plants)*



Silencing level of *LjT36I04*	Uninfected	Infected
	84,4%	53,1%
	84,7%	9,6%
	-	16,3%
	-	-

Figure 6. *Real time PCR results for LjT36I04 gene in selected transformed plants*

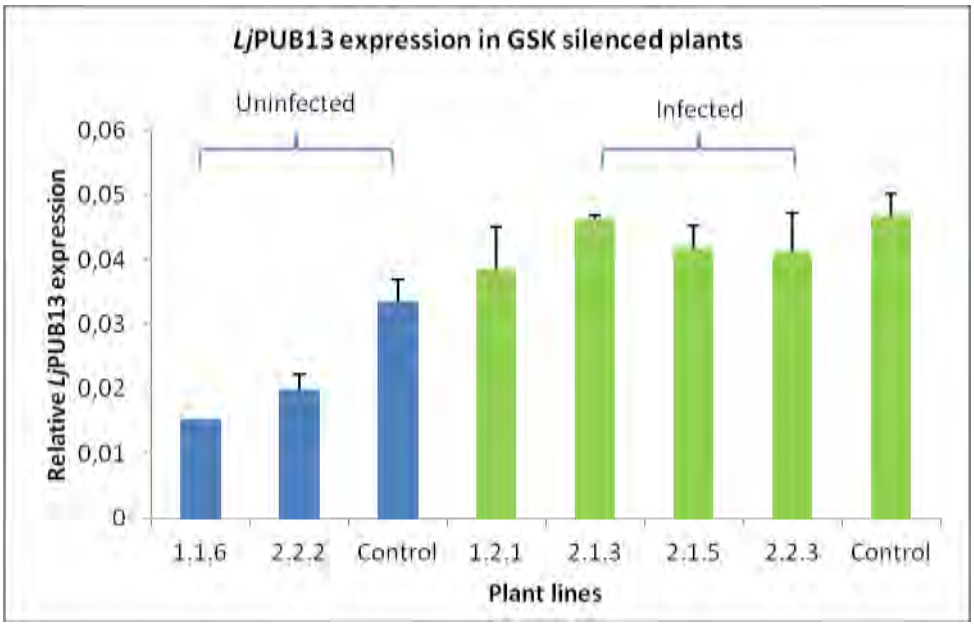


Figure 7. *Real time PCR results for LjPUB13 gene in selected transformed plants.*

4.5. External application of terpenes

The putative role of *LjGSK3β* is thought to be one of a catalytic and/or regulating nature in a pathway that merges cell proliferation and natural compounds such as lupeol and the hypothesis based on the gene expression analysis results that *LjGSK3β* is involved in the early stages of the symbiotic interaction between rhizobium and plants. In order to further examinethis hypothesis ,terpenes such as lupeol, β-amyrin and betulinic acid were applied on the roots in the presence or absence of *M.loti.* Germinated seeds of *Lotus japonicus* were transferred into Jensen medium. Three days later the roots were inoculated with 15μl of *M.loti* and terpene solutions were added. Roots were collected 15 min and 48 hours post inoculation. The sampling was followed by Real Time PCR in order to determine the expression levels of certain genes under the applied conditions. *LjGSK3β* expression was analyzed using t-test and ANOVA and the results are summarized at Table 1.

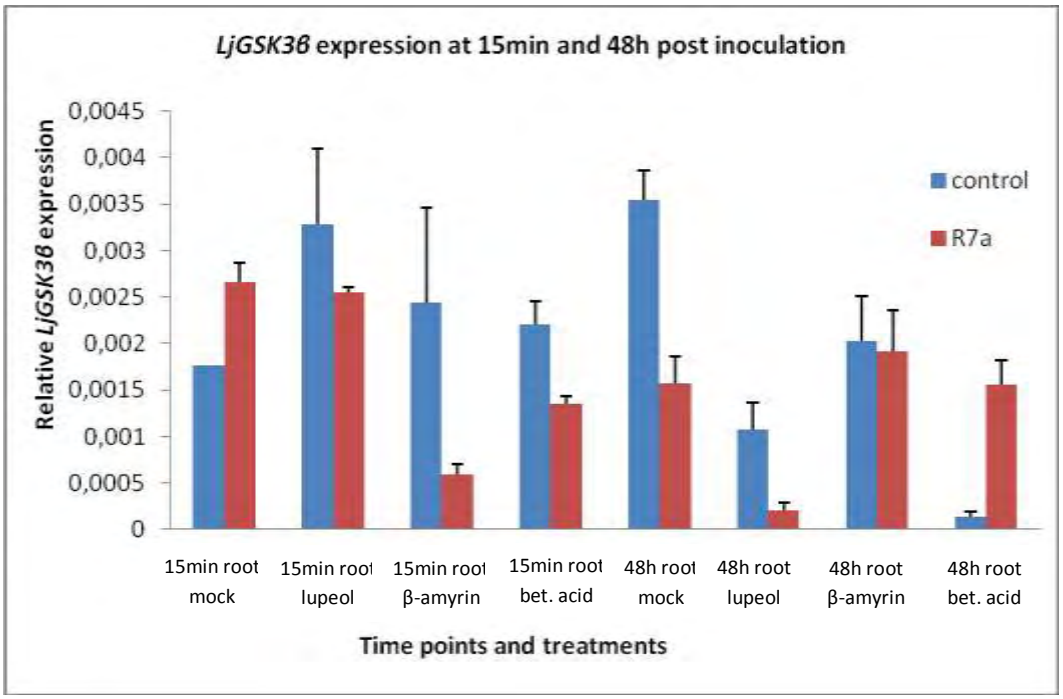


Figure 11. *A collective graph showing the expression levels of LjGSK3β in cDNAs from both time points, infected and uninfected.*

	15min mock	**15min lupeol**	**15min amy**	**15min bet. acid**	**48hours mock**	**48 hours lupeol**	**48 hours amy**	**48 hours bet. acid**
t-test	significant P=0,045	not significant	not significant	significant P=0,031	significant P=0,011	not significant	not significant	significant P=0,048

Table 1. *Comparison using t-test between control and R7A treatments in the same time point e.g. 15min unifected roots treated with lupeol compared to 15min roots infected and treated with lupeol.*

Again after the expression levels of *LjGSK3β* were analyzed, more genes were analysed by real time PCR. This time the cDNAs used were from infected with *M.loti* plants, in an attempt to detect differences between time points while the plant is trying to establish its symbiotic relationship with rhizobium. Statistical analysis followed for each gene separately in an attempt to clarify the relationship between

gene expression, terpene application and maybe the time of tissue sampling after inoculation. The results of the t-test analysis are summarized under each graph.

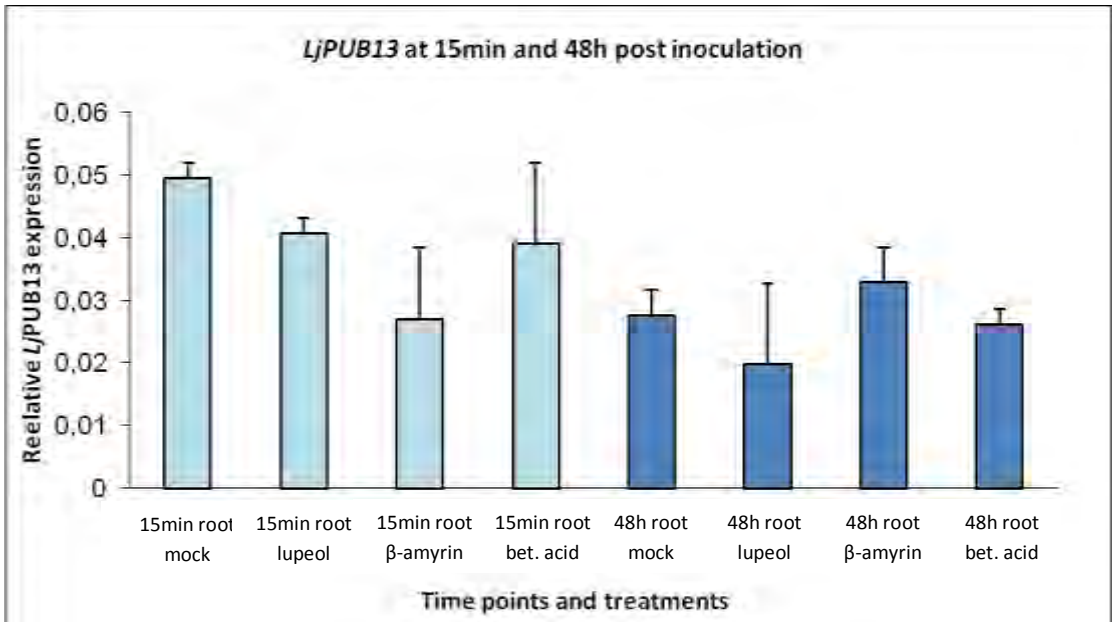


Figure 14. *Expression of LjPUB13 in roots of infected plants 15minutes and 48 hours post inoculation.*

	15min-48h mock	**15min-48h lupeol**	**15min amy**	**15min-48h bet. acid**
t-test	significant P=0,016	not significant	not significant	not significant

Table 2. *Results of t-test p<0,05 for the mean values of LjPUB13 expression in different time points but treated with the same terpenes*.

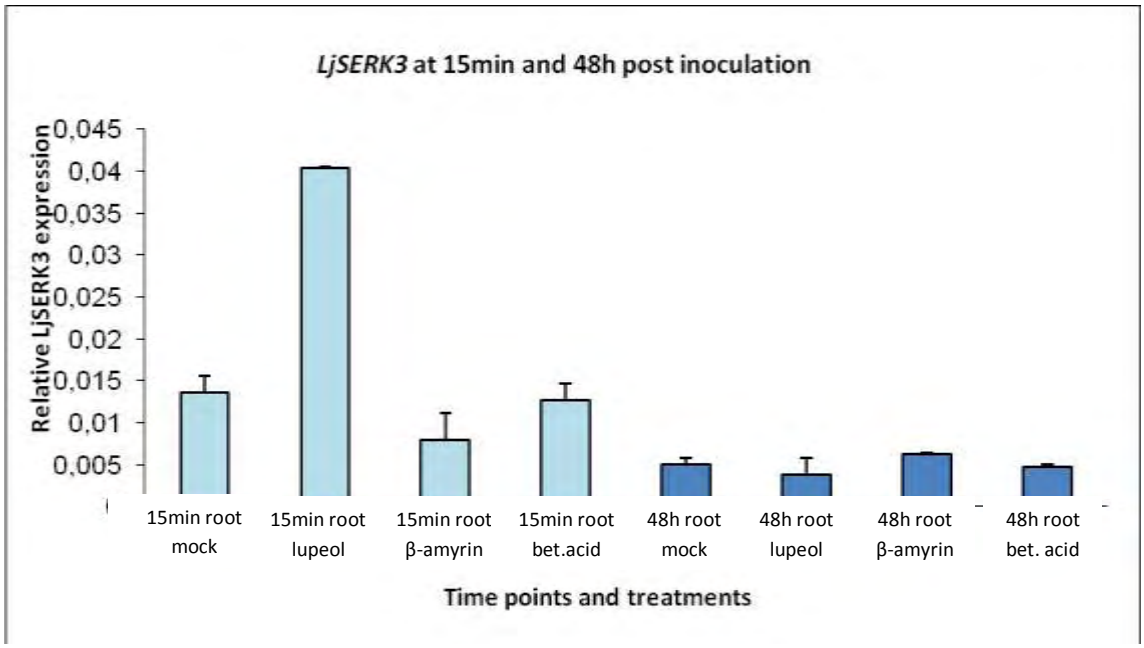


Figure 15. *Expression of SERK in roots of infected plants 15minutes and 48 hours post inoculation.*

	15min-48h mock	**15min-48h lupeol**	**15min-48h amy**	**15min-48h bet. acid**
t-test	significant P=0,046	significant P=0,05	not significant	significant P=0,047

Table 3. *Results of t-test p<0,05 for the meanvalues of LjSERK expression in different time points but treated with the same terpenes*.

For *LjSERK* the two way ANOVA analysis showed that for F(1,15)=8,9, p<0,5 and Fcr= 4,5 different time points do affect the gene expression. But with F(3,15)=0,234, p<0,5 and Fcr(3,15)=3,29 terpenes did not seem to affect the gene expression. Lastly, for F(3,15)=7,7 p<0,5 with Fcr(3,15)=3,29 we reject the hypothesis that different time point and terpenes interaction will have no significant effect.

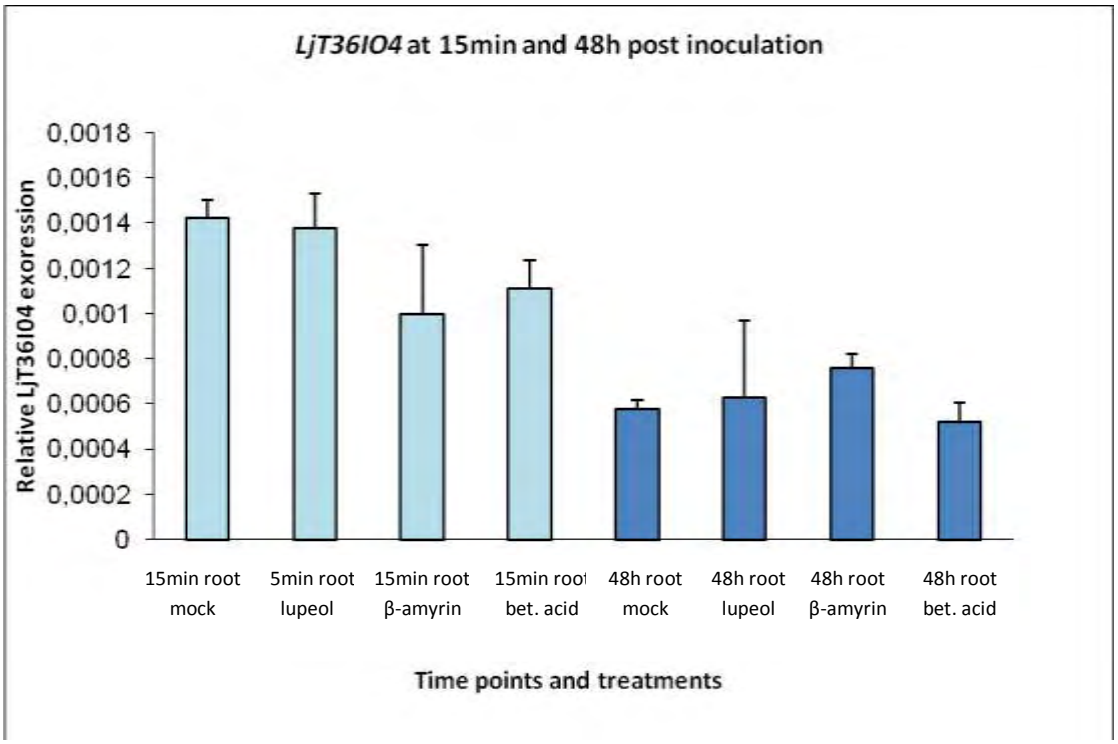


Figure 16. *Expression of LjT36I04 in roots of infected plants 15minutes and 48 hours post inoculation.*

	15min-48h mock	**15min-48h lupeol**	**15min amy**	**15min-48h bet. acid**
t-test	significant P=0,002	not significant	not significant	significant P=0,025

Table 3. *Results of t-test p<0,05 for the meanvalues of LjT36IO4 expression in different time points but treated with the same terpenes*.

For *LjT36I04* the two way ANOVA analysis led to values much greater than the Fcr respectively for every hypothesis thus resulting to their rejection.

4.6. Protein overexpression and purification

One of the aims of this research was to find a molecule that interactswith *LjGSK3β* as well as the downstream effects of this interaction. One hypothesis was the direct binding with the terpene lupeol based on (Harish *et al.,*2008) In order for such

interaction to be proven, *LjGSK3β* needed to be expressed in a specific protein overexpression vector, purified and then crystallized in presence of lupeol. To begin with, *LjGSK3β* gene sequence was inserted in expression vectors pET28a and pGEX-6P. The primers used for the insertion are:

GSK.EcoRI_F	5'-ACGGAATTCATGAACATGATGAGACGG-3'
GSK.Xho_R	5'-AGCTCTCGAGTCAACTCCTTGCATGCTC-3'

A variety of different conditions were applied during the expression experiments. Temperatures varied from 18^{o}C to 37^{o}C, IPTG varied from 0,1-1mM and expression induction varied from 2 to 18 hours. Types of cells used were the DE3 BL21 and Rosetta. For vector pET28a, temperature of 28 ^{o}C and IPTG of 0,5mM for 4 hours and Rosetta cells seem to give enough quantity of protein in order to proceed to the next step. For vector pGEX, temperature of 37 ^{o}C, IPTG of 0,2mM for 4 hours and Rosetta cells, is the condition that produces the greater amount of soluble protein.

Furthermore in order to purify the protein and proceed to crystallization, the soluble sample passed through the FPLC. The protein expressed with pGEX was used for our next experiments.

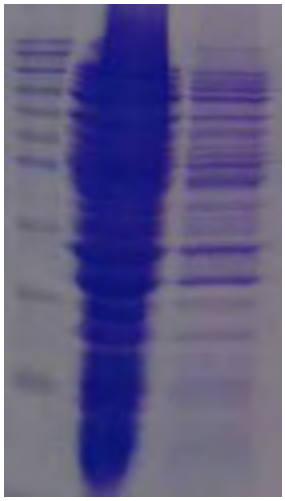


Figure 19. *First column-Ladder, Second column-After induction (37^{o}C,o,2mM IPTG, 4hours) Third column- Supernatant with soluble proteins after sonication*

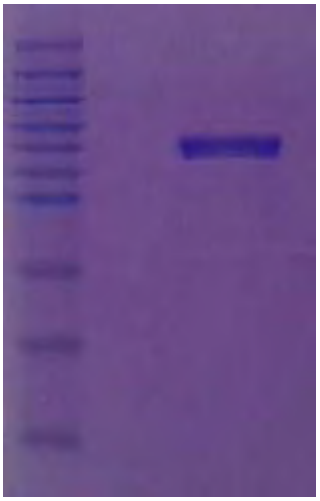


Figure 20. *First column-Ladder Second column-Purified Lj*GSK3β

4.7. Autophosphorylation assay and lupeol

After the overexpression and the purification process it was important to verify that the kinase was indeed active. In order to establish that, we used a coupled assay where we were able to assess the capability of the molecule to phosphorylate itself. Alongside with the verification of the kinase activity, this experiment was held in presence of lupeol in order to give the two molecules a chance for direct interaction. The experiment was repeated two times for lupeol and DMSO in order to verify the repeatability of the results. DMSO was also put through the process as it is the solvent of lupeol and it was important to prove that any effect seen on the kinase is only due to lupeol.

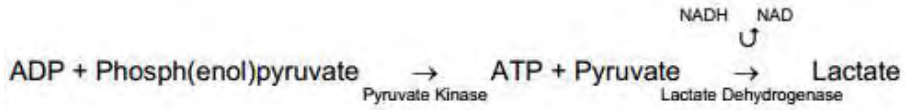


Figure 21. *Depiction of the NADH-linked coupled assay. An event of phopshorylation provides the ADP and then a series of reactions begins having as a result NADH consumption, which is measured as an absorbance decrease in 340nm.*

1st experiment

Sample containing ATP and DMSO and sample containing ATP, *Lj*GSK3β and DMSO show no statistically significant difference in the NADH reduction rate. Also the sample with ATP and lupeol compared to tha sample containing ATP, *Lj*GSK3β and lupeol seems to have no statistically significant difference in the reduction rate of NADH for 0,05 confidence interval. The analysis was based on the t value of the difference between slopes. All the values produced by SPSS are summarized in the Table below the graphs. Values marked in red show no statistical significance.

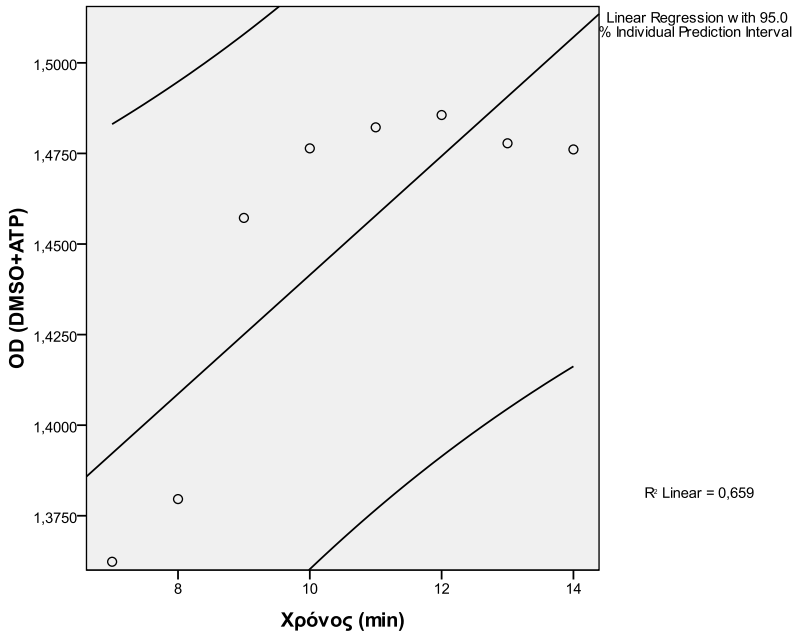


Figure 22. *Selected values of absorbance that present a linear graph in sample containing ATP and DMSO*

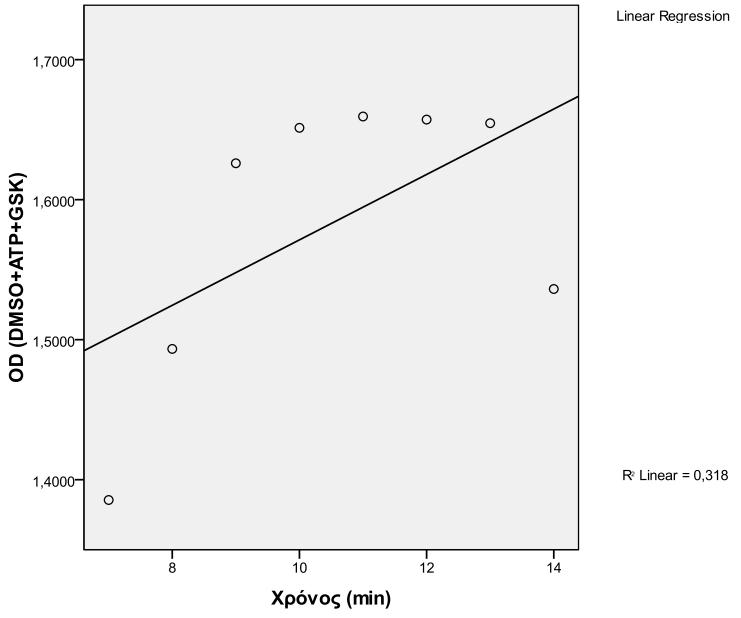


Figure 23. *Selected values of absorbance that present a linear graph in sample containing ATP, Lj*GSK3β *and DMSO*

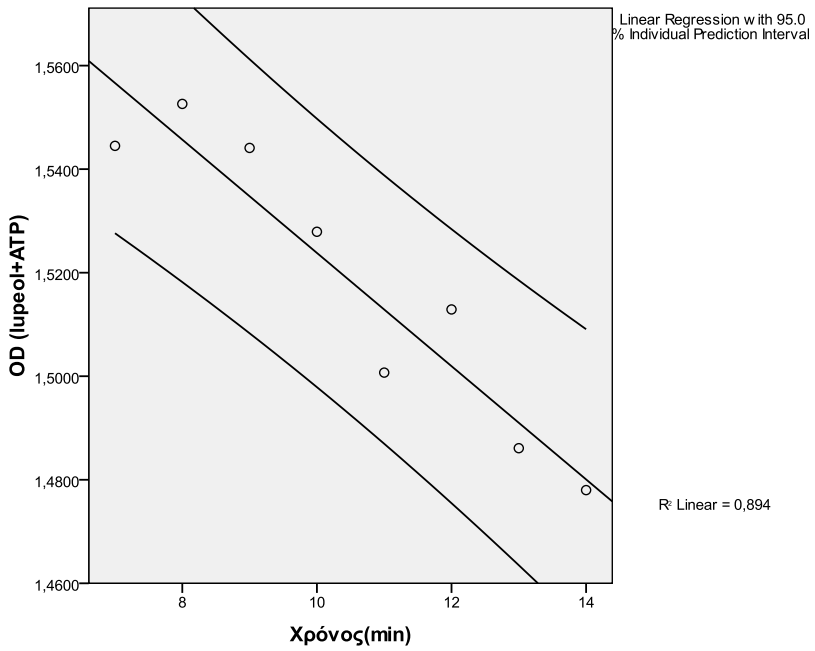


Figure 24. *Selected values of absorbance that present a linear graph in sample containing ATP and lupeol*

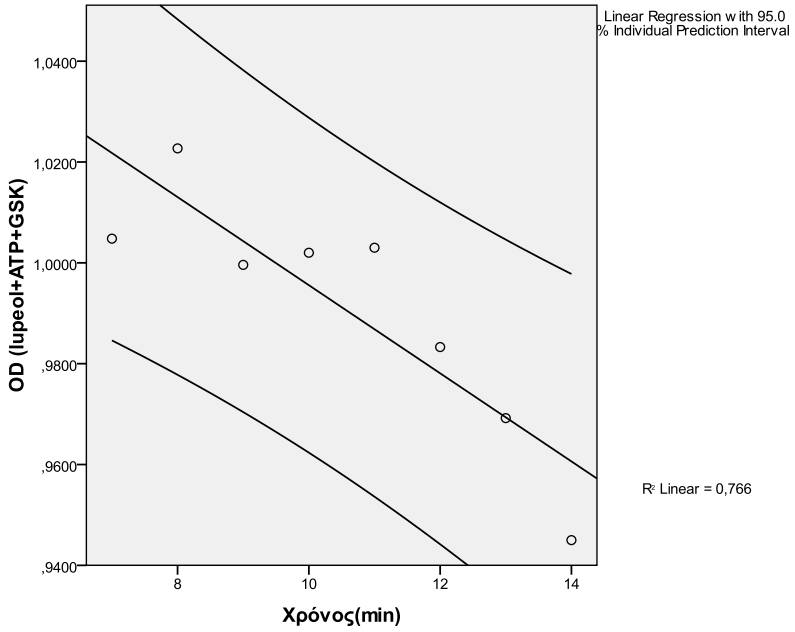


Figure 25. *Selected values of absorbance that present a linear graph in sample containing ATP, Lj*GSK3β *and lupeol.*

Table: The calculations of the significance between the differences of the slopes b2-b3 for the first experiment

Experiment	Sample	b_2	S_{b2}	Sample	b_3	S_{b3}	b_2-b_3	S_{b2-b3}	t_{s2-s3}	df	P
DMSO	DMSO+ATP	0.016	0.005	DMSO+ATP+GSK	0.023	0.014	-0.007	0.024	0.292	12	<0.876
lupeol	lupeol+ATP	-0.011	0.002	lupeol+ATP+GSK	-0.009	0.002	-0.002	0.003	0.666	12	<0.518

2nd experiment

In the second repeat the samples were once again analyzed using the t value of the difference between slopes. This time both treatments (DMSO and lupeol) showed statistically significant difference between the samples containing the kinase and those who did not. Again the confidence interval used was 0.05. The values are summarized in the Table below.

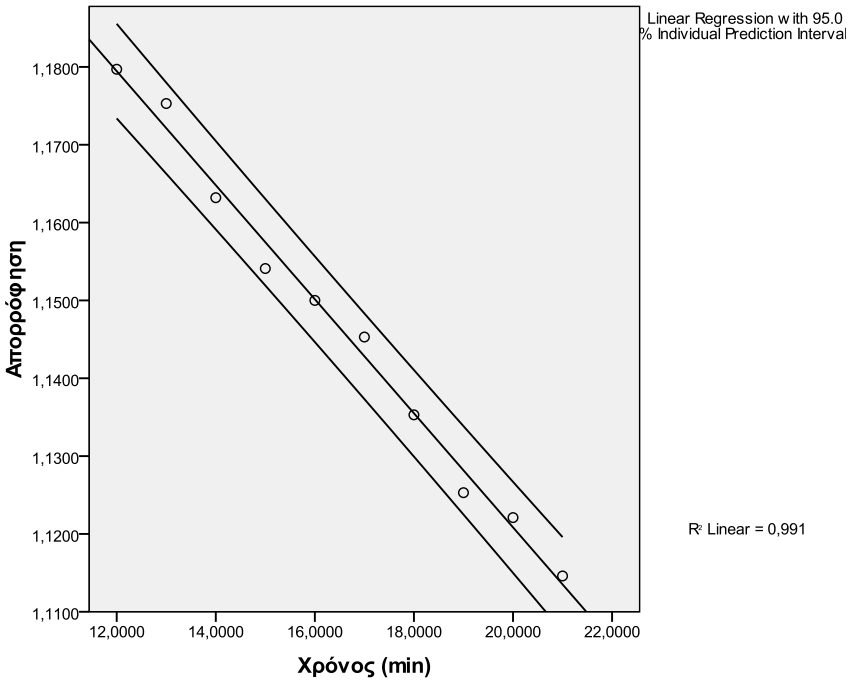


Figure 26. *Selected values of absorbance that present a linear graph in sample containing ATP, Lj*GSK3β *and DMSO*

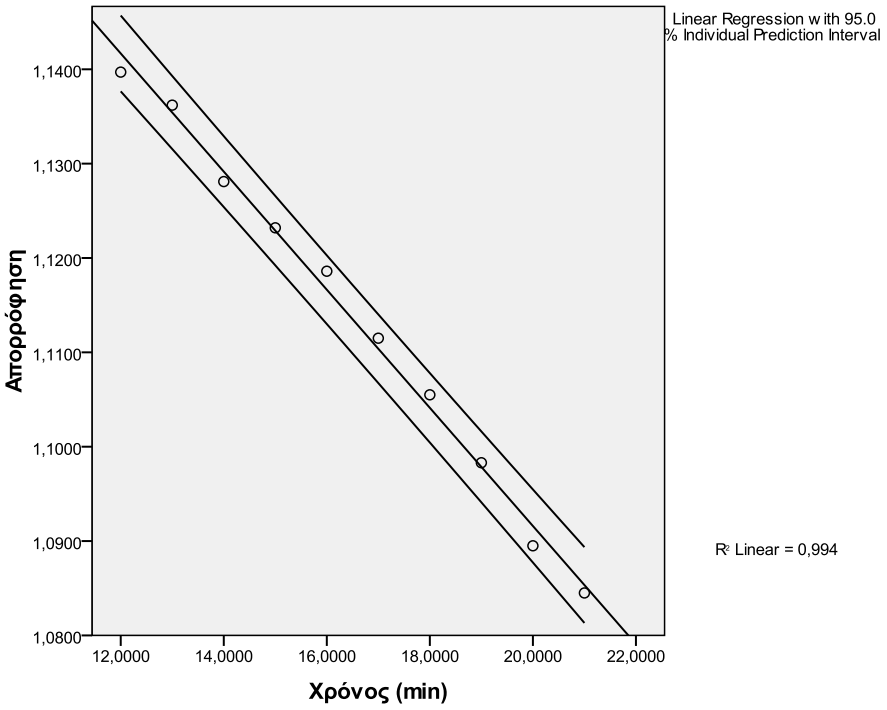


Figure 27. *Selected values of absorbance that present a linear graph in sample containing ATP and DMSO*

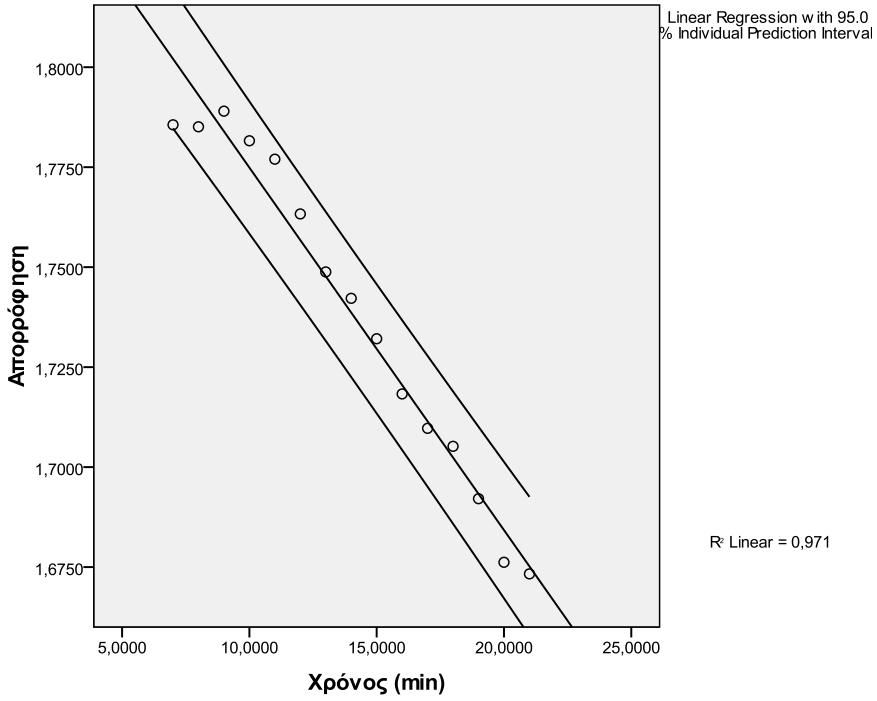


Figure 28. *Selected values of absorbance that present a linear graph in sample containing ATP and lupeol*

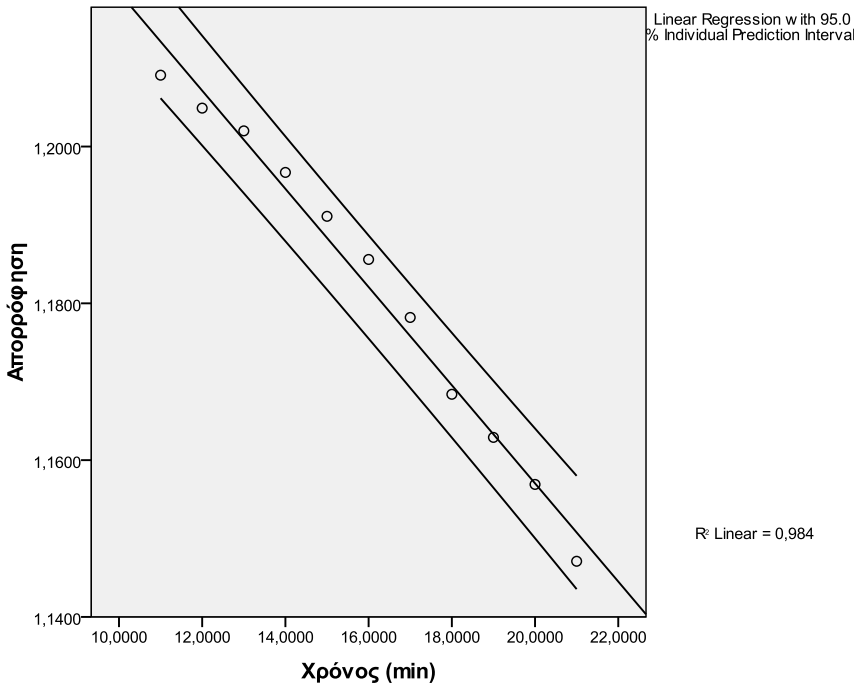


Figure 29. *Selected values of absorbance that present a linear graph in sample containing ATP, Lj*GSK3β *and lupeol*

Table: Calculations of the significance between the differences of the slopes b2-b3

Experiment	Sample	b_2	S_{b2}	Sample	b_3	S_{b3}	b_2-b_3	S_{b2-b3}	t_{b2-b3}	df	P
DMSO	+ATP/-/+DMSO	-0,006	0,0001	+ATP/+GSK/+DMSO	-0,007	0,0001	0,001	0,00014	7,143	16	<0,001
Lupeol	+ATP/-/+lupeol	-0,009	0,0001	+ATP/+GSK/+lupeol	-0,006	0,0001	-0,003	0,00014	21,428	22	<0,001

5. Discussion

Glycogen synthase kinases are proven to intervene in the regulation of important and basic cell functions in mammals as well as in plants. The importance of these proteins in cell processes such as cell differentiation and proliferation, glycogen metabolism and immunological responses are adequately documented in mammals through various studies (Grimes and Jope *et al.,* 2001; Jope, *et al.*, 2007). Also except functional analysis of GSK3β there are studies that have marked the kinase as the target of various natural and synthetic compounds ultimately aiming in the therapeutic effect of GSK3β on a wide range of diseases. The same elaborate knowledge of GSK3β though, does not apply in the field of plant systems where the study of GSK-like molecules and their function is considered a newly emerging field. In one of the first papers reviewing the Shaggy-like kinases of plants by Jonak and Hirt *et al.,* 2002, it becomes obvious how little is known about plant GSKs. The most studied molecules are the *Arabidopsis thaliana* shaggy-like kinases which seem to hold a key role in the modulation of various processes such as hormone signaling (Yan et al., 2009) and flower development (Dornelas *et al.,* 2000). In another model-plant, *Lotus japonicus*, even fewer are known about the activity of shaggy–like kinases. *L. japonicus* is known to possess two sequences that belong to Ser/Thr GSK3 family kinases (Kameshita *et al.,* 2005). The aim of this study was the isolation and characterization of one GSK3β kinase in *L. japonicus* and its functional analysis concerning procedures such as symbiotic relationship establishment and interaction with natural compounds known as terpenes. Initially we located the full length sequences of the two GSK3β-like kinases in Kazusa database. The first gene isolated and amplified was *LjGSK3β,* a homologue of *AtSKϑ* of *Arabidopsis thaliana*, which was recently identified as a part of brassinosteroid signaling (Rozhon *et al.,* 2010). The second gene isolated was *LjT36I04*, a gene homologue to *AtSKα*, which is studied for its role in redox stress responses (Dal Santo *et al.,* 2012). One of the most important processes that take place during the life of *L. japonicus* is its symbiotic relationship with bacteria. In order to control possible association between the expression of the two kinases and the establishment of symbiosis, we studied the

expression of *LjGSK3β* and *LjT36I04* in roots infected with rhizobium *M.loti* at 15 minutes and 48 hours after inoculation. The choice of time points was made based on a previous experiment where the expression of *LjGSK3β* was studied at 1 hour and 7 hours post inoculation making us choose a wider range of time points. Quantitative real time PCR analysis of both experiments showed that the expression of *LjGSK3β* is two times higher in infected roots compared to the uninfected. Terpenes such as lupeol, β-amyrin and betulinic acid were applied to the roots of specific plants. Expression analysis revealed that at 15minutes post inoculation *LjGSK3β* transcript levels are increased whereas at 48 hours there is a statistically significant decrease. Furthermore, lupeol is shown to dowregulate the *LjGSK3β* expression levels at 48 hours post inoculation in both experiments. B-amyrin shows no effect on *LjGSK3β* while betulinic acid seems to have an opposite effect at different time points compared to the inoculation-caused effect, meaning it decreases *LjGSK3β* expression at 15 minutes when it is increased in untreated plants and it induces *LjGSK3β* expression at 48 hours when it is decreased in untreated plants. *LjT36I04* expression seems to be lower at 48 hours post inoculation in general and the terpene with the greater effect on *LjT36I04* expression is betulinic acid which seems to downregulate the gene in both 15 minutes and 48 hours post inoculation. The various expression patterns of *LjGSK3β* between uninfected and infected plants have led us to focus on the role on *LjGSK3β.* That gene seems to be induced in presence of other organisms such as *M.loti* suggesting a role in innate immunity pathway. Key molecules in that pathway are the receptors BAK1/SERK3 and FLS2 and also PUB13, an E3 ubiquitin ligase recently identified in the laboratory (Tsikou, unpublished data). Specifically, in *A. thaliana* the kinase-receptor BAK1/SERK3 forms a complex with FLS2 in presence of flagellin while it simultaneously phosphorylates *At*PUB13. This phosphorylation is a prerequisite for FLS2-PUB13 interaction (Chinichilla *et al.,* 2007; Lu *et al.,* 2011). Following these data, *LjPUB13* and *LjSERK3* genes were also analysed in the tissues from all the previous experiments. *LjPUB13* expression showed a significant decrease at 48 hours post inoculation while none of the terpenes affected the levels. *LjSERK3* exhibited also a significant decrease at 48 hours post inoculation only on this gene lupeol and betulinic acid seem to have a negative effect. Moreover, in an attempt to clarify the

role of *Lj*GSK3β and *Lj*T36I04, transformed plants with *LjGSK3β* silenced and inoculated with *M.loti* were prepared. The outcome of this experiment was complex since not only *LjGSK3β* was silenced but also *LjT36I04.* The two genes have an 89% identity making it sometimes difficult to separate by appropriate primer selection. Nevertheless *LjPUB13* in silenced plants was upregulated in presence of rhizobium. The next step for the characterization of *Lj*GSK3β was to try and prove the direct interaction between lupeol and *Lj*GSK3β that is documented on mammals. Two approaches were designed, one of them involves a photometric assay in order to monitor the kinase activity in presence of lupeol and the other the co-crystallization of *Lj*GSK3β protein and lupeol in order to prove the direct binding of the molecules. Both approaches were successful in mammal GSK3β (Dajani *et al.,* 2001; Harish *et al.,* 2008; Haar *et al.,* 2001). Results of the autophosphorylation assay indicate that when lupeol is added then a reduction in the kinase activity is observed even though that result is still not verified. As for the crystallization experiment, effort is being made to achieve high concentrations of pure *Lj*GSK3β which is needed for the completion of the experiment. Although the results of the above mentioned experiments are not yet conclusive, they can function as a prime indication of *Lj*GSK3β and propably *Lj*T36I04 involvement in the early stages of establishing the symbiotic relationship between legumes and rhizobia. Furthermore, the similarities between *Lj*GSK3β and *At*SKθ may also suggest a more complicated role of the kinase in a cross-talk of immunity and brassinosteroid signaling pathways (Albrecht et al., 2012).

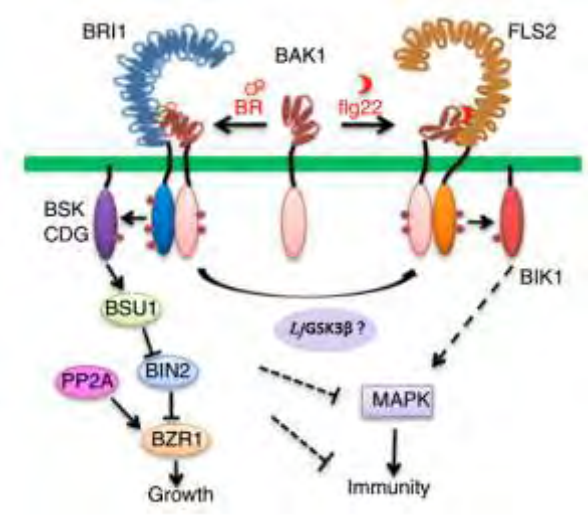


Image by Wang *et al.,* 2012

Alignments

LjGSK3b-AskTheta protein alignment in Uniprot

Identity 76.17%

Identical positions 361

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1    MNVMRRLKSIASGRTSISSDPGGDYALKRAKLDQENDNLCVDPMQVDQNSSCFEMKADVL   60  Q96287   KSG8_ARATH
1    MNMMRRLKSIASGRTSISSDPGGDSNSKRAKLDQETEKKVNEETKTLGGKD-QEQ-HVDA   58  Q53VM1   Q53VM1_LOTJA
     **:**********************   ********.::   :  :.   ..  *

61   SQESVAGTSNVPAV--SEKPVDDQLPDVMIEMKIRDERNANREDKDMETTVVNGSGTETG  118  Q96287   KSG8_ARATH
59   SKESTVGTSDVSTVAKTEKSGFDELPKELHEMKIKDEKSKNNNEKDIEASIVSGNGTETG  118  Q53VM1   Q53VM1_LOTJA
     *:**..***:* :*  :**   *:**. : ****:**:. *.::**:*:::*.*.*****

119  QVITTTVGGRDGKPKQTISYMAQRVVGTGSFGVVFQAKCLETGEQVAIKKVLQDKRYKNR  178  Q96287   KSG8_ARATH
119  QIITTAIGGRDGQPKQTISYMAERVVGTGSFGVVFQAKCLETGEAVAIKKVLQDKRYKNR  178  Q53VM1   Q53VM1_LOTJA
     *:***::*****:**********:********************* ***************

179  ELQIMRLQDHPNVVRLRHSFFSTTDKDELYLNLVLEYVPETVYRASKHYTKMNQHMPIIF  238  Q96287   KSG8_ARATH
179  ELQVMRTVDHPNIVKLKHCFFSTTDKDELYLNLVLEFVPETVYKVSKQYIRVHQHMPIIY  238  Q53VM1   Q53VM1_LOTJA
     ***:**  ****:*:*:*.*****************:******:.**:* ::.******:

239  VQLYTYQICRALNYLHRVVGVCHRDIKPQNLLVNPQTHQLKICDFGSAKMLVPGEPNISY  298  Q96287   KSG8_ARATH
239  VQLYIYQICRALNYLHQVIGVCHRDIKPQNLLVNPQTHQLKICDFGSAKMLVPGEPNISY  298  Q53VM1   Q53VM1_LOTJA
     **** ***********:*:*****************************************

299  ICSRYYRAPELIFGATEYTNAIDMWSGGCVMAELLLGQPLFPGESGIDQLVEIIKILGTP  358  Q96287   KSG8_ARATH
299  ICSRYYRAPELIFGATEYTTAIDMWSVGCVLAELLLGHPLFPGESGVDQLVEIIKVLGTP  358  Q53VM1   Q53VM1_LOTJA
     ******************.****** ***:******:********:********:****

359  TREEIRCMNPNYTEFKFPQIKAHPWHKIFHKRMPPEAVDLVSRLLQYSPNLRCTALEACA  418  Q96287   KSG8_ARATH
359  TREEIRCMNPHYNEFKFPQIKAHPWHKVFYKRMPPEAVDLVSRLLQYSPNLRCTALAACA  418  Q53VM1   Q53VM1_LOTJA
     **********.*.***************:*:************************** ***

419  HPFFDDLRDPNVSLPNGRALPPLFNFTAQELAGASTELRQRLIPAHCQGTGSSS  472  Q96287   KSG8_ARATH
419  HPFFNDLRDPNASLPNGQPLPPLFNFTPEELAHAPDELRLRLIPEHARS-----  467  Q53VM1   Q53VM1_LOTJA
     ****:******.*****: ******** :*** *  *** **** *.:.
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Human GSK3b-LjGSK3B using **Uniprot**

Identity 46.825%

Identical positions 236

```
1    MNMMRRLKSIASGRTSISSDPGGDSNSKRAKLDQETEKKVNEETKTLGGKDQEQHVDASK   60  Q53VM1   Q53VM1_LOTJA
1    ----------------------------------------------MSGRPR--------    6  P49841   GSK3B_HUMAN
                                                     :.*: :

61   ESTVGTSDVSTVAKTEKSGFDELPKELHEMKIKDEKSKNNNEKDIEASIVSGNGTETGQI  120  Q53VM1   Q53VM1_LOTJA
7    --------TTSFA--ESCKPVQQPSAFGSMKVSRDK----------------DGSKVTT-   39  P49841   GSK3B_HUMAN
             .::.*  *..   : *. : .**:. :*                 :*::.

121  ITTAIGGRDGQPKQTISYMAERVVGTGSFGVVFQAKCLETGEAVAIKKVLQDKRYKNREL  180  Q53VM1   Q53VM1_LOTJA
40   -VVATPGQGPDRPQEVSYTDTKVIGNGSFGVVYQAKLCDSGELVAIKKVLQDKRFKNREL   98  P49841   GSK3B_HUMAN
      ..*  *:  :  * :**   :*:*.******:***  ::** ***********:*****

181  QVMRTVDHPNIVKLKHCFFSTT-DKDELYLNLVLEFVPETVYKVSKQYIRVHQHMPIIYV  239  Q53VM1   Q53VM1_LOTJA
99   QIMRKLDHCNIVRLRYFFYSSGEKKDEVYLNLVLDYVPETVYRVARHYSRAKQTLPVIYV  158  P49841   GSK3B_HUMAN
     *:**.:** ***:*:: *:*:  .***:******::******:*:::* *.:* :*:***

240  QLYIYQICRALNYLHQVIGVCHRDIKPQNLLVNPQTHQLKICDFGSAKMLVPGEPNISYI  299  Q53VM1   Q53VM1_LOTJA
159  KLYMYQLFRSLAYIHS-FGICHRDIKPQNLLLDPDTAVLKLCDFGSAKQLVRGEPNVSYI  217  P49841   GSK3B_HUMAN
     :**:**: *:* *:*. :*:***********::*:*  **:******* ** ****:***

300  CSRYYRAPELIFGATEYTTAIDMWSVGCVLAELLLGHPLFPGESGVDQLVEIIKVLGTPT  359  Q53VM1   Q53VM1_LOTJA
218  CSRYYRAPELIFGATDYTSSIDVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPT  277  P49841   GSK3B_HUMAN
     ****************:**::**:**.**********:*:***:*****************

360  REEIRCMNPHYNEFKFPQIKAHPWHKVFYKRMPPEAVDLVSRLLQYSPNLRCTALAACAH  419  Q53VM1   Q53VM1_LOTJA
278  REQIREMNPNYTEFKFPQIKAHPWTKVFRPRTPPEAIALCSRLLEYTPTARLTPLEACAH  337  P49841   GSK3B_HUMAN
     **:** ***.*.************ ***  * ****: * ****:*:*. * * * ****

420  PFFNDLRDPNASLPNGQPLPPLFNFTPEELAHAPDELRLRLIPEHARS------------  467  Q53VM1   Q53VM1_LOTJA
338  SFFDELRDPNVKLPNGRDTPALFNFTTQELSSNPPLAT-ILIPPHARIQAAASTPTNATA  396  P49841   GSK3B_HUMAN
      **::*****..****:  * ***** :**:  *      *** ***

468  ------------------------  467  Q53VM1   Q53VM1_LOTJA
397  ASDANTGDRGQTNNAASASASNST  420  P49841   GSK3B_HUMAN
```

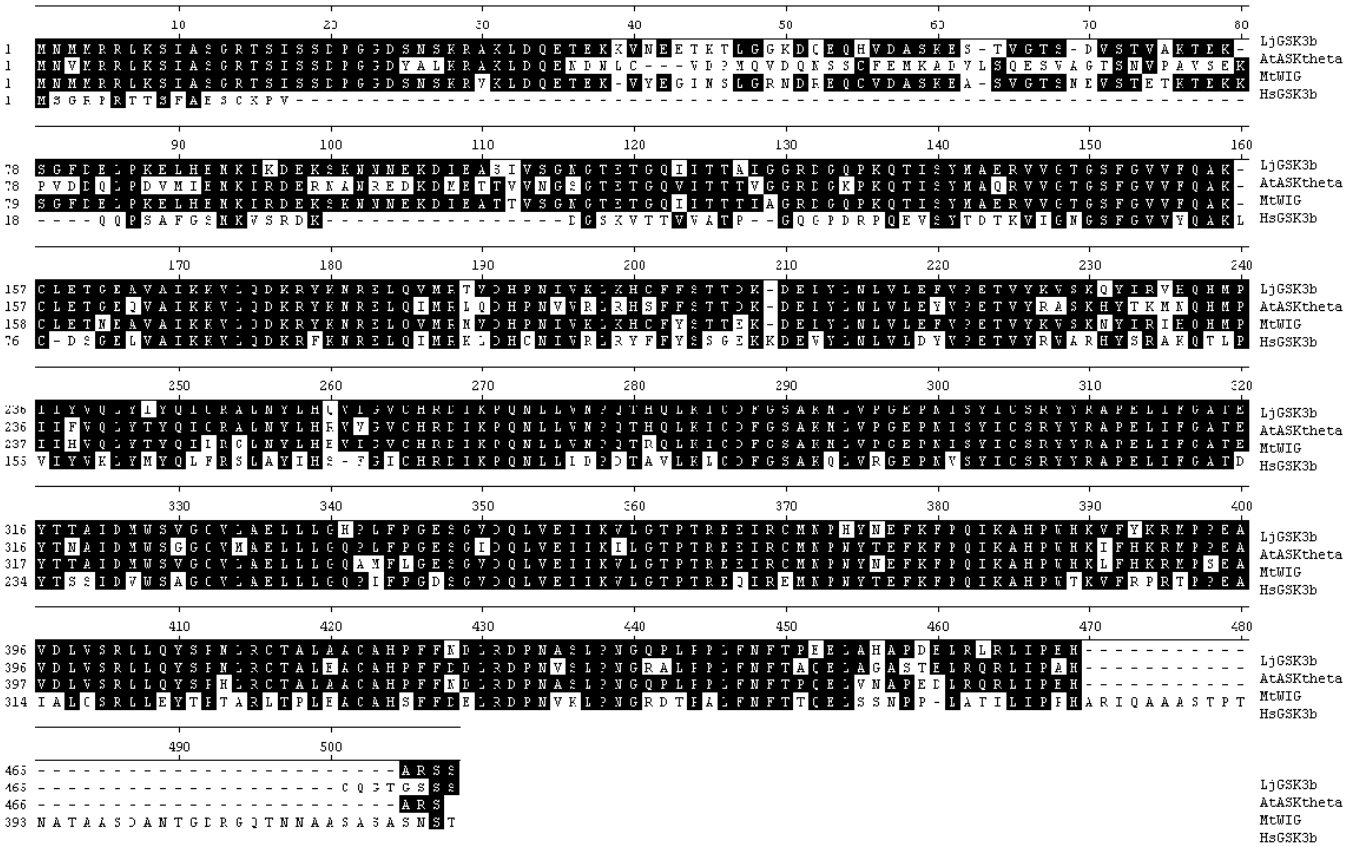
***WIG medicago-LjGSK3b using* Uniprot**

Identity 88.06%

Identical positions 413

```
1    MNMMRRLKSIASGRTSISSDPGGDSNSKRAKLDQETEKKVNEETKTLGGKDQEQHVDASK   60  Q53VM1   Q53VM1_LOTJA
1    MNMMRRLKSIASGRTSISSDPGGDSNSKRVKLDQETEK-VYEGINSLGRNDREQCVDASK   59  Q9FSG4   Q9FSG4_MEDSA
     ****************************.******* * *  ::** :*:** *****

61   ESTVGTSDVST--VAKTEKSGFDELPKELHEMKIKDEKSKNNNEKDIEASIVSGNGTETG  118  Q53VM1   Q53VM1_LOTJA
60   EASVGTSNEVSTETKTEKKSGFDELPKELHEMKIRDEKSKNNNEKDIEATTVSGNGTETG  119  Q9FSG4   Q9FSG4_MEDSA
     *::****:  :  . . :****************:**************: *********

119  QIITTAIGGRDGQPKQTISYMAERVVGTGSFGVVFQAKCLETGEAVAIKKVLQDKRYKNR  178  Q53VM1   Q53VM1_LOTJA
120  QIITTTIAGRDGQPKQTISYMAERVVGTGSFGVVFQAKCLETNEAVAIKKVLQDKRYKNR  179  Q9FSG4   Q9FSG4_MEDSA
     *****:*.********************************** *****************

179  ELQVMRTVDHPNIVKLKHCFFSTTDKDELYLNLVLEFVPETVYKVSKQYIRVHQHMPIIY  238  Q53VM1   Q53VM1_LOTJA
180  ELQVMRMVDHPNIVKLKHCFYSTTEKDELYLNLVLEFVPETVYKVSKNYIRIHQHMPIIH  239  Q9FSG4   Q9FSG4_MEDSA
     ****** *************:***:***********************:***:*******:

239  VQLYIYQICRALNYLHQVIGVCHRDIKPQNLLVNPQTHQLKICDFGSAKMLVPGEPNISY  298  Q53VM1   Q53VM1_LOTJA
240  VQLYTYQILRGLNYLHEVIGVCHRDIKPQNLLVNPQTRQLKICDFGSAKMLVPGEPNISY  299  Q9FSG4   Q9FSG4_MEDSA
     **** *** *.*****:*******************:**********************

299  ICSRYYRAPELIFGATEYTTAIDMWSVGCVLAELLLGHPLFPGESGVDQLVEIIKVLGTP  358  Q53VM1   Q53VM1_LOTJA
300  ICSRYYRAPELIFGATEYTTAIDMWSVGCVLAELLLGQAMFLGESGVDQLVEIIKVLGTP  359  Q9FSG4   Q9FSG4_MEDSA
     ************************************: :* ******************

359  TREEIRCMNPHYNEFKFPQIKAHPWHKVFYKRMPPEAVDLVSRLLQYSPNLRCTALAACA  418  Q53VM1   Q53VM1_LOTJA
360  TREEIRCMNPNYNEFKFPQIKAHPWHKLFHKRMPSEAVDLVSRLLQYSPHLRCTALAACA  419  Q9FSG4   Q9FSG4_MEDSA
     **********.****************:*:**** ***************.**********

419  HPFFNDLRDPNASLPNGQPLPPLFNFTPEELAHAPDELRLRLIPEHARS  467  Q53VM1   Q53VM1_LOTJA
420  HPFFNDLRDPNASLPNGQPLPPLFNFTPQELVNAPEDLRQRLIPEHARS  468  Q9FSG4   Q9FSG4_MEDSA
     ****************************:**..**::** *********
```

Alignment of *LjGSK3β,ASKϑ, MtWIG* and *HsGSK3β* using Megalign.



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46. Harish, B. G. *et al.* Wound healing activity and docking of glycogen-synthase-kinase-3-beta-protein with isolated triterpenoid lupeol in rats. *Phytomedicine* **15,** 763–767 (2008).

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